AZUREOMYCINS A AND B, NEW CELL WALL PEPTIDOGLYCAN SYNTHESIS INHIBITORS FROM PSEUDONOCARDIA AZUREA NOV. SP.

DISSERTATION

zur Erlangung des Grades eines Doktors

der Naturwissenschaften

der Fakultät für Biologie

der Eberhard-Karls-Universität Tübingen

vorgelegt von

PRISKA SPIRI-NAKAGAWA

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Associate Professor TANAKA contributed his untiring support and many valuable discussions.

The data on the chemistry of azureomycins A and B, as well as many items for discussion were contributed by Mr. Y. TANAKA. The producing strain was described by Mrs. Y. TAKAHASHI and Miss K. MATSUYAMA. Miss S. SUZUKI contributed her valuable technical assistance during a part of the *in vitro* experiments.

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Abbreviations used in the text:

AM	aerial mycelium
Dpm	diaminopimelic acid
DTT	dithiothreitol
GlcNAc	N-acetylglucosamine
MIC	minimal inhibition concentration
Mol.wt.	molecular weight
MP	melting point
rpm	revolutions per minute
Tris	tris(hydroxymethyl)aminomethane
TTC	triphenyltetrazolium chloride
UDP-GlcNAc	uridine 5'-diphosphate-N-acetyl-glucosamine
UDP-MurNAc	uridine 5'-diphosphate-N-acetylmuramyl
UDP-MurNAc-pentapeptide	uridine 5'-diphosphate-N-acetylmuramyl-L-alanyl-D-glutamyl-meso-diamino-pimelyl-D-alanyl-D-alanine
UMP	uridine 5'-monophosphate
v/v	volume per volume

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1. INTRODUCTION

The cell wall peptidoglycan, also known as murein or mucopeptide, is a unique structure of the procaryotes. It occurs in all Gram-positive and Gram-negative organisms except Halobacterium and Mycoplasma which do not need the protection of this mesh-like "bag-shaped macromolecule"[1] because of their peculiar environments.

Peptidoglycan is composed of glycan and peptide subunits, as can be derived from the name. The two sugars, N-acetylmuramic acid and N-acetylglucosamine form long heteropolysaccharide chains which are cross-linked by peptides attached to the N-acetylmuramic acid. Whereas these sugar molecules are very common in various bacteria, there exist, however, variations of the amino acid composition of the peptide bridge and the mode of cross-linking[2,3]. Mannomuramic acid of the glycan of Micrococcus lysodeicticus is a rare special type of sugar[4].

The occurrence of peptidoglycan in the cell wall of the procaryotes alone, is of great importance for the therapy of bacterial infections with antibiotics. In general, inhibitors which interfere with the biosynthesis of this procaryote-specific macromolecule[5], have a very low toxicity in humans. Among them, penicillins, cephalosporins[6], D-cycloserine[7] and phosphonomycin[8] are currently used in clinical medicine.

For animal growth promotion, another field where antibiotics are frequently used, moenomycin[9,10], macarbomycin[11], bacitracin[12] and enduracidin[13] are very important.

As, however, more and more bacterial strains become resistant against these antibiotics, additional new compounds active against the resistant strains are necessary to overcome infectious diseases. For the treatment of superinfections and opportunistic infections which impose serious problems recently, effective new inhibitors of low toxicity are needed, too.

Therefore, a screening system for new inhibitors of bacterial cell wall peptidoglycan was developed in the laboratories of Kitasato University, School of Pharmaceutical Sciences, Microbial Chemistry and The Kitasato Institute (Prof. Dr. S. ŌMURA)[14]. This screening system was based on the three following tests:

1) insensitivity of Mycoplasma[15], which lacks peptidoglycan[16,17], to these inhibitors.
2) selective inhibition of the incorporation of labeled Dpm into acid-insoluble fraction in a Dpm-requiring Bacillus.
3) passage of the culture broth through a membrane filter to obtain inhibitors with molecular weights lower than 1000.

In the course of this screening program, new antibiotics, azureomycins A and B, were discovered in the culture

broth of Pseudonocardia azurea nov. sp.[18,19]. They consist of sugar and amino acid moieties and phenolic chromophores, and are active against Gram-positive bacteria.

The present work deals with the morphological characterization of the producing strain, with the isolation and characterization of azureomycins A and B, and with the studies on the mode of action of these new antibiotics[20,21]. The results of the investigation of the morphological characterization of the new strain and the isolation and characterization of the antibiotics are described in part one of this thesis. Parts two and three deal with the mode of action in vivo and in vitro, respectively.

This is the first complete communication on the new antibiotics azureomycins A and B. The peculiar purpose to investigate into the molecular mode of inhibitory action, was preceeded by research under the guidance of Professor Dr. S. ŌMURA with participation of Associate Professor Dr. H. TANAKA and Mrs. R. ŌIWA (screening program), Mr. Y. TANAKA (chemical characterization of azureomycins), Mrs. Y. TAKAHASHI and Miss K. MATSUYAMA (description of the producing strain).

Parts of the results on azureomycins and the producing microorganism have been submitted or prepared for publication in scientific journals during the course of preparation of the present thesis[14,18-21] including the valid description of the producing strain Pseudonocardia azurea TAKAHASHI et ŌMURA.

2. MATERIALS AND METHODS

2.1. Materials and methods for the morphological characterization of the producing strain, and for the production, isolation and characterization of azureomycins A and B

2.1.1. Morphological characterization of the producing strain

The morphology of the strain was observed microscopically. For the photomicrographs, an Olympus Vanox photomicroscope was used. The electronmicrographs were taken with a scanning electron microscope, model JEM-100C ASJD-4D (Nihon Denshi).

2.1.2. Production of azureomycins A and B

Pseudonocardia azurea isolated from the original strain AM-3696 by a conventional monospore isolation technique (monospore isolate No. 16), was used for the production of azureomycins A and B. Spores and mycelia of this monospore isolate grown on an agar slant (1 % glucose, 0.5 % peptone, 0.5 % meat extract, 0.3 % NaCl and 1.5 % agar, pH 7.0, adjusted prior to autoclaving) were transferred in a 500 ml Sakaguchi's flask containing

100 ml of a seed medium (2 % glucose, 2 % soybean meal, 0.3 % NaCl, pH 7.0) and cultivated for 2 days at 27° C. The seed culture (600 ml) was transferred into a 50-liter jar fermentor containing 30 liters of a fermentation medium composed of 2 % dextrin, 1 % soybean meal, 0.3 % yeast extract, 0.15 % K_2HPO_4, 0.05 % KH_2PO_4, 0.1 % $MgSO_4 \cdot 7H_2O$ (pH 7.0) and was cultivated for 4 days at 27° C with agitation of 250 rpm and with aeration of 15 liters/minute. The antibiotic activity in the culture filtrate was monitored by a paper disc method, using Bacillus subtilis PCI 219 as a test organism and glucose minimal agar (0.7 % K_2HPO_4, 0.2 % KH_2PO_4, 0.05 % Na-citrate, 0.1 % $(NH_4)_2SO_4$, 0.01 % $MgSO_4 \cdot 7H_2O$, 0.2 % glucose, 1 - 1.5 % agar, pH 6.8) as a test medium.

Large scale fermentation was carried out using a 1000-liter tank fermentor containing 600 liters of the fermentation medium described above, at the Laboratories of Pharmaceutical Science, Kyowa Hakko Kogyo Co., Ltd., Japan.

2.1.3. Isolation of azureomycins A and B

The antibiotic in the culture filtrate (600 l) was adsorbed on carbon and eluted from the carbon cake with 50 % acetone containing 0.01 N HCl. The active eluate was concentrated and the concentrate was desalted by a batch-

wise treatment with barium hydroxide and subsequent filtration. The filtrate was passed through a column of Amberlite IRC-50 (H^+-form, 10 l). The active fractions eluted from the column with 0.1 N HCl were collected, desalted with a carbon column and eluted as described above. The active eluate from the carbon column was neutralized with a slurry of Amberlite IR-45 (OH^--form) and filtered, then lyophilized to give a crude powder (170 g, containing ca. 1 % antibiotically active substances). A part of the crude powder (160 g) was purified by successive column chromatographies on carbon (1.1 liter), cellulose (Avicel, 800 ml) and carboxymethylcellulose (CM-cellulose, H^+-form, 370 ml) and separated into two components, peak A and peak B by CM-cellulose. The fractions of both peaks were further purified by Biogel P-2 and Avicel column chromatographies and desalted with carbon columns. The eluates from the carbon columns were concentrated and to the concentrates ten volumes of acetone were added. The floccose precipitates were collected by centrifugation, dissolved in a small volume of water and lyophilized to give purified powders of azureomycins A and B as their hydrochlorides.

Although not always mentioned, desalting steps were included between each column chromatography. The desalting was performed by adsorption of the active substance on a carbon column and elution from it with 50 % acetone containing 0.01 N HCl, and by a subsequent treatment with a slurry of Amberlite IR-45 (OH^--form) followed by filtration.

2.1.4. Characterization of azureomycins A and B

2.1.4.1. Physico-chemical characterization

Spectra

The ultraviolet absorption spectra were taken with a Shimadzu double beam spectrophotometer model 210 A, the infrared spectra in KBr with a Jasco IRA-1 diffraction grating infrared spectrometer. $E^{1\%}_{1cm}$ was determined with a Hitachi spectrophotometer model 100-40.

Determination of melting points

A Mitamura Riken melting point determinator was used.

Thin layer chromatography

Avicel and silica gel thin layer chromatography were used to identify the antibiotics and to examine their purity[22].

2.1.4.2. Biological characterization

Minimal inhibitory concentrations (MIC)

MIC were determined by the serial agar dilution method (described in 23)) using heart infusion agar for aerobic bacteria (37° C, 20 hrs), Gifu anaerobic medium for anaerobic bacteria (37° C, 48 hrs) and potato-glucose agar for fungi (27° C, 48 hrs).

Bioautography

After thin layer chromatography, the plates were dried, 1 x 25 cm adhesive tapes were attached to it and transferred to a 32 x 24 cm big test plate containing spores of the test organism B. subtilis PCI 219. The adhesive tapes were taken off and the plate was incubated at 37° C overnight. After incubation, the R_f values of the antibiotics could be determined according to the place of the inhibition zone.

2.2. Materials and methods for the study of the mode of action of azureomycin B in vivo

2.2.1. Bacterial strain

Bacillus cereus T which was obtained from Dr. K. IZAKI, School of Agriculture, Tohoku University, Sendai, Japan, and kept on heart infusion agar slants, was used.

2.2.2. Growth of the bacterial strain

B. cereus T was grown at 37° C in nutrient broth (1 % peptone, 1 % meat extract, 0.5 % NaCl, pH 7.2 before autoclaving at 120° C for 15 minutes). In small scale experiments (up to 10 ml culture volume) a Jasco Bio-Log II

photometer and a Monod shaker were used for the incubation. Large scale growth was done with a reciprocal shaker (Takasaki Kagaku Kikai Co., Ltd., Japan) using Sakaguchi's flasks.

For viable counts, an aliquot of the growing culture in biophotometer cells was diluted with water, spread on a nutrient agar plate and incubated at 37° C overnight.

The seed (1 %) for all cultures was an overnight culture of 14 hours incubation.

2.2.3. Microscopical investigation

B. cereus T was grown in nutrient broth at 37° C (5 ml) in a Monod tube until the absorbance at 660 nm reached 0.4 (the absorbance was measured with a Coleman Junior II spectrophotometer). Then azureomycin B was added to give a final concentration of 20 µg/ml and incubated for 30 minutes. A culture without antibiotic served as control. Then a loopful of the cultures (control and antibiotic-treated ones) was transferred to microscope slides, stained with a 0.2 % phenolfuchsin solution and observed with an Olympus Vanox photomicroscope.

2.2.4. Incorporation of [^{3}H]Dpm, [^{14}C]glucosamine, [^{3}H]-thymidine, [^{3}H]uridine or [^{14}C]leucine into acid-insoluble macromolecular fraction of growing cells of B. cereus T

The method according to SIDDHIKOL et al.[24] was modified and employed here.

B. cereus T was incubated in nutrient broth in a Monod tube until the absorbance at 660 nm reached 0.2. Azureomycin B and [^{3}H]thymidine (2 µCi), [^{3}H]uridine (0.5 µCi), L-[^{14}C]leucine (2 µCi), 2,6-[^{3}H]Dpm (0.2 µCi) or [^{14}C]glucosamine (2 µCi) were added to 1.4 ml of the culture in the case of [^{3}H]thymidine incorporation and to 1.6 ml in the latter ones. Cold thymidine solution (0.2 ml of a 1 µg/ml-solution) was then added to the reaction mixture containing [^{3}H]thymidine. The reaction mixtures, total volume 2 ml each, were incubated in Monod tubes. After 5, 10, 15 and 20 minutes, 0.4 ml aliquots of the cultures were put into 10 ml of ice-cold 5 % trichloroacetic acid; the resulting acid-insoluble precipitates were collected on a Millipore filter (pore size 0.22 µm) and washed twice with 10 ml of cold 5 % trichloroacetic acid. The radioactivities of the acid-insoluble fractions on the filters were counted with 10 ml of a liquid scintillation fluid (4 g 2,5-diphenyloxazole, 100 mg 1,4-bis-(2-(5-phenyloxazolyl)) benzene in 1 l toluene) using an Aloka LSC-651 scintillation counter. The counting efficiency was 48 % in the case of [^{3}H] and 88 % for [^{14}C].

2.2.5. Peptidoglycan synthesis and nucleotide accumulation in a growing culture

For the time course experiment of peptidoglycan synthesis and nucleotide accumulation, B. cereus T was incubated in 4.5 ml nutrient broth in a Monod tube until the absorbance at 660 nm reached 0.6 (half maximal growth). Then 2,6-[^{3}H]Dpm (5 µCi) and azureomycin B (50 µg/ml, final concentration) were added to give a total volume of 5 ml. After 10, 30 and 60 minutes, 0.2 ml aliquots of the culture were given into 10 ml of ice-cold 5 % trichloroacetic acid, the acid-insoluble precipitates were collected on a Millipore filter (pore size 0.22 µm) and the radioactivities were counted (corresponding to peptidoglycan synthesis). Simultaneously, 1 ml aliquots of the culture were centrifuged for 10 minutes at 1500 x g at 4° C. The pellets were washed twice with water, suspended in 1 ml water and heated for two minutes in boiling water. The heated suspensions were centifuged for 10 minutes at 1500 x g, and 0.2 ml aliquots of the supernatants were chromatographed on Toyo Roshi No. 50 paper (1 x 40 cm) with isobutyric acid : 1 N ammonia (5 : 3, v/v) for 16 hours. The paper strips were cut into 1 x 2 cm pieces and the radioactivities counted in the toluene scintillation system described under 2.2.4. In the above solvent system, the R_f value of UDP-MurNAc-peptides is about 0.15. The counting efficiency for the Millipore filters was 48 % and for the Toyo Roshi No. 50 paper 9.7 %.

2.2.6. Identification of the nucleotide accumulated in the cells

B. cereus T was incubated in 1 l nutrient broth distributed to 100 ml in 10 Sakaguchi's flasks. At half maximal growth of the culture (absorbance at 660 nm was 0.6), 500 ml of it was treated with azureomycin B (20 µg/ml, final concentration) for 30 minutes. The nontreated half served as control. Extraction of the nucleotide precursor and anion-exchange chromatography were carried out according to STROMINGER[25] in the following way. The cells, control and antibiotic-treated ones, were harvested by centrifugation at 1500 x g for 10 minutes at 6° C and washed once with 200 ml water and an additional two times with 100 ml water each. The wet cell pastes were suspended in 16 ml water each, 16 ml of ice-cold 10 % trichloroacetic acid was added and the nucleotides extracted overnight at 4° C. Then, the cell fragments were removed by centrifugation at 1500 x g for 10 minutes. The extracts were treated 5 times with an equal volume of ether to remove trichloroacetic acid and concentrated to 15 ml each. The extracts were then adjusted to pH 8.6 with 2 N NaOH and applied to a Dowex-1 x 2 (Cl-type) column (12 x 1.2 cm) and eluted step-wise with the following solvents: A, 0.002 N HCl; B, 0.01 N HCl; C, 0.05 M NaCl in 0.01 N HCl; D, 0.1 M NaCl in 0.01 N HCl. The flow rate was 10 ml/15 minutes and fractions of 10 ml were collected. Elution of the

nucleotides was monitored by measuring the absorbance at 260 nm of each fraction with a Hitachi spectrophotometer model 100-40. The elution patterns of the extracts of control and azureomycin B-treated culture were compared and the UV-absorption peak, obtained only in the case of antibiotic-treatment, was further purified by charcoal (10 ml) adsorption and elution with 50 % ethanol in 0.5 N ammonia, and paper chromatography on Toyo Roshi No. 51 A paper in the solvent system isobutyric acid : 0.5 N ammonia (5 : 3, v/v). After elution of the UV-absorbing band from the paper with water at 100° C for 30 minutes, this material was analyzed qualitatively and quantitatively as follows: uridine, spectrophotometrically; GlcNAc, as N-acetylhexosamine described by REISSIG et al.[26] and STROMINGER[25]; amino acids, by amino acid analyzer after acid hydrolysis with 6 N HCl in a sealed tube at 110° C for 15 hours and removement of the hydrochloric acid.

2.3. Materials and methods for the study of the mode of action *in vitro*

2.3.1. Bacterial strains

Bacillus megaterium KM and *Escherichia coli* Y-10 used for the preparation of particulate enzymes were obtained from Dr. P. E. REYNOLDS, Department of Biochemistry, Uni-

versity of Cambridge, U. K. and Dr. K. IZAKI, School of Agriculture, Tohoku University, Sendai, Japan, respectively. B. cereus T as described under 2.2.1. was used for the preparation of UDP-MurNAc-pentapeptide. All strains were kept on heart infusion agar slants.

2.3.2. Preparation of particulate enzymes from B. megaterium KM and E. coli Y-10

The method described by OKA[27] was employed here.

2.3.2.1. Growth of bacterial strains

B. megaterium KM was grown at 37° C in a 50-liter jar fermentor with 250 rpm agitation and 30 liters/minute aeration. The culture volume was 30 liters. The culture broth contained 1 % peptone, 1 % meat extract, 0.5 % yeast extract and 0.5 % NaCl and was adjusted to pH 7.0 before autoclaving. An overnight culture (14 hours incubation) grown in the same medium was used as seed culture. The inoculum size was 1.3 %. The cells were harvested by centrifugation at an optical density of 0.85 at 660 nm and washed once with 0.01 M Tris-HCl buffer (pH 7.0) containing 0.8 % NaCl and 10^{-3} M $MgCl_2$. The washed wet cell paste (190 g) obtained was divided into 20 g-portions and stored at -80° C.

E. coli Y-10 was grown at 37° C in 20 Sakaguchi's flasks containing 100 ml each of a medium composed of 1 % meat extract, 1 % peptone, 0.5 % yeast extract, pH 7.0. An overnight culture grown in the same medium was used as seed culture. The inoculum size was 1 %. The cells were harvested as described before for the B. megaterium KM cells. Eleven grams of wet cell paste were obtained and stored at -80° C.

2.3.2.2. Preparation of particulate enzymes

All steps were carried out at 4° C. Twenty grams of the wet cell paste of B. megaterium KM were mixed with the same amount of alumina powder and ground in a mortar with a pestle for 15 minutes. Thirty ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 10^{-3} M $MgCl_2$ and 10^{-3} M DTT was added to the homogenate. To remove alumina, the mixture was first centrifuged at 1500 x g for 15 minutes. The supernatant was centrifuged at 8000 x g for 10 minutes to remove cell debris and unbroken cells. The resulting supernatant was centrifuged at 100,000 x g for 30 minutes to sediment the membranes. These were washed once by the buffer described above and then suspended in 1 ml of this buffer.

The preparation of the E. coli Y-10 particulate enzyme was carried out as described above for that of B. megaterium KM, suspended at a protein concentration of

49 mg/ml, as measured by the method of LOWRY et al.[28], and stored at -80° C.

2.3.2.3. UMP-treatment of the B. megaterium KM particulate enzyme

The procedure described by HAMMES et al.[29] for the Staphylococcus aureus Copenhagen particulate enzyme was used with modifications in the way that the here described reaction mixture did not contain KCl but did DTT. The B. megaterium KM particulate fraction was known to need no K^+ but a SH-group protecting reagent for optimal activity, in contrast to the S. aureus Copenhagen particulate enzyme[30]. The reaction mixture for UMP-treatment contained: 1 ml of a suspension of particulate enzyme from B. megaterium KM, obtained as described under 2.3.2.2., and 5.5 ml of 0.05 M Tris-HCl buffer (pH 7.8) containing 4×10^{-5} M UMP·Na, 0.042 M $MgCl_2$ and 10^{-3} M DTT. The incubation was performed at 25° C for 10 minutes.

Phospho-MurNAc-pentapeptide, contained in the membrane fraction as undecaprenol-P-P-MurNAc-pentapeptide is removed from the membranes by the formation of UDP-MurNAc-pentapeptide, a reaction which is catalyzed by the phospho-MurNAc-pentapeptide translocase in the presence of an excess of UMP (see also Fig. 24 (1) in "Discussion"). The UDP-MurNAc-pentapeptide is water-soluble and can be se-

parated from the particulate fraction by centrifugation. To remove UDP-MurNAc-pentapeptide efficiently, the membranes were washed 4 times with 0.05 M Tris-HCl (pH 7.5) containing 10^{-3} M $MgCl_2$ and 10^{-3} M DTT. Then they were suspended in 1 ml of this buffer at a protein concentration of 21.5 mg/ml as measured by the method of LOWRY et al.[28], devided into 200 µl-portions and stored in small test-tubes at -80° C. At this temperature no loss of activity was observed after two months.

2.3.3. Substrates for the *in vitro* reaction of lipid intermediate accumulation and peptidoglycan synthesis

UDP-[^{3}H]GlcNAc (6.6 Ci/mmole) and UDP-[^{14}C]GlcNAc (235 mCi/mmole) were purchased from New England Nuclear, Boston, Mass., U. S. A. UDP-MurNAc-L-Ala-D-Glu-*meso*[^{3}H]-Dpm-D-Ala-D-Ala (11.6 µCi/µmole) was a generous gift from Dr. T. OKA, Tokyo Research Laboratories of Kyowa Hakko Kogyo, Co., Ltd. UDP-GlcNAc was purchased from Sigma Chemical Company, St. Louis, U. S. A. UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm-D-Ala-D-Ala was obtained essentially as described by REYNOLDS[31] and OKA[27] for the preparation of *meso*-Dpm-labeled UDP-MurNAc-pentapeptide in the following way. The bacterial strain used for the intracellular accumulation was *B. cereus* T, grown in 1 l nutrient broth (1 % peptone,

1 % meat extract, 0.5 % yeast extract, pH 7.2) supplemented with L-lysine (200 mg/l) and Dpm (meso, LL, DD) (25 mg/l), at 37° C to an absorbance of 0.6 at 660 nm. The cells were harvested by centrifugation (1500 x g for 10 minutes at 10° C) and washed three times with 0.01 M potassium-phosphate buffer (pH 7.0) containing 10^{-3} M $MgCl_2$, 0.8 % NaCl and 20 µg/ml chloramphenicol. The washed cells were then resuspended in 250 ml of a medium containing (in g/l): KCl 4, $MgCl_2 \cdot 6H_2O$ 4, NH_4Cl 2, Na_2SO_4 0.15, $Na_2HPO_4 \cdot 12\ H_2O$ 0.35, glucose (autoclaved separately) 10, uracil 0.04, L-alanine 0.125, L-glutamic acid 0.125, L-lysine 0.2, Dpm 0.025, vancomycin 0.02 and chloramphenicol 0.05, pH 7.2. The incubation in this medium at 37° C was 30 minutes. The cells were harvested by centrifugation (1500 x g for 10 minutes at 4° C), and the nucleotide precursor which accumulated in the cells was extracted with ice-cold 5 % trichloroacetic acid (80 ml) overnight and purified by Dowex-1 x 2-column chromatography according to STROMINGER[25], charcoal adsorption and elution followed by paper chromatography. This purification procedure was the same as described for the in vivo experiment (2.2.6.).

2.3.4. Assay for lipid intermediate accumulation and peptidoglycan synthesis.

This assay measures the incorporation of P-MurNAc-[^{3}H]pentapeptide from UDP-MurNAc-[^{3}H]pentapeptide (reaction

mixture A), of [^{3}H]GlcNAc from UDP-[^{3}H]GlcNAc (reaction mixtures B and D) or of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc (reaction mixture C) into lipid intermediate and peptidoglycan.

Reaction mixture A, which was similar to that one used by TANAKA et al.[32], contained the following components in a total volume of 30 µl:

1) 3.3×10^{-4} M UDP-MurNAc-L-Ala-D-Glu-meso-[^{3}H]Dpm-D-Ala-D-Ala (11.6 µCi/µmole)
2) 3.3×10^{-4} M UDP-GlcNAc
3) 2.5×10^{-2} M $MgCl_2$
4) 1.0×10^{-3} M DTT
5) 0.25 M Tris-HCl buffer (pH 8.5)
6) 10 µl of particulate enzyme from B. megaterium KM (125 µg of protein)
7) antibiotic or water

The incubation was performed at 25° C for 30 minutes.

The standard reaction mixture B was composed of the following compounds in a total volume of 40 µl:

1) 3.75×10^{-6} M UDP-MurNAc-L-Ala-D-Glu-meso-Dpm-D-Ala-D-Ala
2) 3.75×10^{-7} M UDP-[^{3}H]GlcNAc (6.6 Ci/mmole; 0.1 µCi)
3) 1.9×10^{-2} M $MgCl_2$
4) 1.0×10^{-3} M DTT
5) 0.19 M Tris-HCl buffer (pH 8.5)

6) 10 µl of particulate enzyme from B. megaterium KM (13.4 µg of protein)

7) antibiotic or water

Deviations from this standard reaction mixture are described under the concerning figures. The incubation period was between 10 and 120 minutes at 25° C.

Reaction mixture C contained the following compounds in a total volume of 40 µl:

1) 1.87×10^{-5} M UDP-MurNAc-L-Ala-D-Glu-meso-Dpm-D-Ala-D-Ala

2) 2.15×10^{-6} M UDP-[^{14}C]GlcNAc (235 mCi/mmole; 0.02 µCi)

3) 1.9×10^{-3} M $MgCl_2$

4) 1.0×10^{-3} M DTT

5) 0.19 M Tris-HCl buffer (pH 8.5)

6) 10 µl of particulate enzyme from E. coli Y-10 (196 µg of protein)

7) antibiotics or water

The incubation period was 2 hours at 25° C.

The reaction mixture D with purified cell walls in a total volume of 50 µl contained:

1) 3×10^{-6} M UDP-MurNAc-pentapeptide

2) 3×10^{-7} M UDP-[^{3}H]GlcNAc (6.6 Ci/mmole; 0.1 µCi)

3) 0.015 M $MgCl_2$

4) 8×10^{-4} M DTT

5) 0.15 M Tris-HCl buffer (pH 8.5)

6) 10 µl of a cell wall-suspension from B. megaterium KM (4.5 mg/ml; 180 µg dry weight/assay tube)

7) 10 µl of particulate enzyme from B. megaterium KM (13.4 µg of protein)

8) antibiotics or water

The incubation period was 2 hours at 25° C.

In the cases of reaction mixtures A, B and D, lipid intermediate and peptidoglycan were determined according to TANAKA et al.[32]. The reactions were stopped by addition of 20 µl of 6 M pyridinium acetate (pH 4.2) and the lipid intermediates were extracted twice with 100 µl of water-saturated n-butanol. The extracts were transferred into a scintillation vial, dried up and the radioactivities counted with an Aloka LSC-651 scintillation counter using the same toluene sintillation fluid as described under 2.2.4. The counting efficiency was 50 %.

Peptidoglycan remaining in the water layer was precipitated by addition of 200 µl of 5 % trichloroacetic acid. The precipitate was collected on a Millipore filter (pore size 0.45 µm) and washed twice with 10 ml of 5 % trichloroacetic acid. The radioactivity of the precipitate on the filter was covered with the toluene scintillation fluid described under 2.2.4. and counted in a LSC. The counting efficiency was 48 %.

In case of reaction mixture C, the separation of lipid intermediate and peptidoglycan was carried out chromatographically.

To stop the reaction, the tubes were placed in boiling water for 1 minute and 25 µl aliquots of each sample were spotted on Toyo Roshi 51 A paper (20 x 30 cm) and chromatographed in the solvent system (isobutyric acid : 1 N ammonia 5 : 3) for 16 hours. After complete removement of the isobutyric acid by washing the paper in acetone, an autoradiogram was prepared using a Kodak X-Omat R film. The film was developed after one week. The radioactive spots on the paper were excised and counted in the toluene scintillation system described under 2.2.4. in an Aloka LSC-651 scintillation counter. The counting efficiency was 90 %.

2.3.5. Isolation of cell walls from B. megaterium KM

The method described by STRUVE et al.[33] was slightly modified. The cells were disintegrated by grinding with alumina; then the unbroken cells were removed by centrifugation (1500xg for 10 min.). The cell walls were sedimented at 9800 x g for 20 minutes. This precipitate was suspended in 0.05 M Tris-HCl (pH 7.5) containing 10^{-3} M $MgCl_2$ and further purified by centrifugation between 1500 x g for 10 minutes and 9800xg for 20 minutes. This process was re-

peated twice and the resulting white precipitate was extracted with 0.5 % Triton X-100 at 2° C for 30 minutes to remove lipids.

After Triton X-100 extraction, the walls were washed 4 times with 0.05 M Tris-HCl buffer (pH 7.5) containing 10^{-3} M $MgCl_2$ and additionally two times with water, and finally suspended in 1.0 ml of water (18 mg dry weight per ml). The purity of the cell wall preparation was checked as described by SALTON[34], for the absence of yellow pigmentation and nucleic acid. The amino acid analysis of the acid-hydrolyzate of the purified walls revealed only alanine, glutamic acid and Dpm in significant quantities.

2.3.6. Assay for the binding of azureomycin B to cell walls from B. megaterium KM

This assay was based on the absorption of azureomycin B at 280 nm and performed in a similar way as described by BEST et al.[35] for vancomycin.

Fig. 1 shows a standard curve of the azureomycin B absorption at 280 nm.

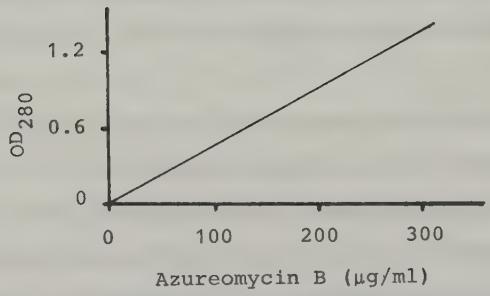


Fig. 1 Standard curve for azureomycin B (HCl salt) in distilled water. Absorbance at 280 nm was measured against a distilled water blank.

Azureomycin B (450 µg) was dissolved in 2.9 ml water and mixed with 600 µg/0.1 ml of a cell wall suspension from B. megaterium KM. After incubation of the mixture for 3 minutes, it was given on a Millipore filter (0.45 µm pore size), the cell walls were filtered off and the UV-absorbance of the filtrate, containing azureomycin B which was not bound to the walls, was measured with a Hitachi spectrophotometer model 100-40.

The effect of Mg^{++} on the binding of azureomycin B and vancomycin to B. megaterium KM cell walls was studied by a procedure similar to that described by SINHA et al.[36]. The assay mixture contained: 0.05 M Tris-HCl (pH 7.8), 10 mM $MgCl_2$, cell walls (150 or 300 µg) and azureomycin B (188 µg) or vancomycin (188 µg) in a total volume of 1.25 ml. The assay was performed at 25° C for 30 minutes.

The cell walls were removed by centrifugation and the UV-absorbance of the supernatant was measured.

The control experiments without cell walls showed that azureomycin B passed the Millipore filter and was not sedimented by centrifugation.

2.4. Summarized alphabetical list of chemicals, radiochemicals and medium components

All chemicals used were guaranteed grade. Not mentioned ones were from Kanto Chemical Co., Inc., Tokyo, Japan.

Table 1

Product	Source
N-Acetyl-D-glucosamine (GlcNAc)	Tokyo Kasei Kogyo Co., Ltd., Japan
Actinomycin D	Daiichi Seiyaku Co., Ltd., Japan
L-Alanine	Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan
Aluminium oxide	Wako Pure Chemical Ind., Ltd., Osaka, Japan
Amberlite IR-45	Organo Co., Ltd., Tokyo, Japan
Amberlite IRC-50	Organo Co., Ltd., Tokyo, Japan

Continuation of Table 1

Product	Source
Avicel	Funakoshi Yakuhin Co., Ltd., Tokyo, Japan
Bacto agar	Difco Lab., Detroit, U. S. A.
Biogel P-2 (100-200 mesh, wet)	Bio-Rad Lab., Richmond, California, U. S. A.
1,4-bis-(2-(5-phenoxazolyl)) benzene (POPOP)	Wako Pure Chemical Ind., Ltd., Osaka, Japan
Carboxymethyl-cellulose (CM-cellulose)	W. and R. Balston, Ltd., U. K.
Charcoal	Takeda Yakuhin Kogyo Co., Ltd., Osaka, Japan
Chloramphenicol	Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan
DL-α,ε-Diaminopimelic acid (meso, LL, DD) (Dpm)	Sigma Chem. Co., St. Louis, U. S. A.
(DL + meso)-2,6-Diamino[6-^{3}H]-pimelic acid dihydrochloride (1.5 Ci/mmole) ([^{3}H]Dpm)	The Radiochemical Centre Amersham, U. K.
2,5-Diphenyloxazole (PPO)	Wako Pure Chemical Ind., Ltd., Osaka, Japan
Dithiothreitol (DTT, Cleland's reagent)	Wako Pure Chemical Ind., Ltd., Osaka, Japan
Dowex-1 x 2 (200-400 mesh)	Muromachi Kagaku Kogyo, Ltd., Tokyo, Japan
Gifu anaerobic medium	Nissui Seiyaku Co., Ltd., Tokyo, Japan

Continuation of Table 1

Product	Source
[^{14}C]Glucosamine (56.5 mCi/mmole) ([^{14}C]GlcN)	The Radiochemical Centre Amersham, U. K.
Glucose	Chugai Seiyaku Kogyo, Co., Ltd., Tokyo, Japan
L-Glutamic acid	Kyowa Hakko Kogyo, Co., Ltd., Tokyo, Japan
Heart infusion agar	Nissui Seiyaku Co., Ltd., Tokyo, Japan
L-[U-^{14}C]Leucine (354 mCi/mmole)	The Radiochemical Centre, Amersham, U. K.
L-Lysine	Kyowa Hakko Kogyo, Co., Ltd., Tokyo, Japan
Meat extract	Kyokuto Seiyaku Kogyo, Co., Ltd., Tokyo, Japan
Peptone	Kyokuto Seiyaku Kogyo, Co., Ltd., Tokyo, Japan
Rifampicin	Daiichi Seiyaku Co., Ltd., Tokyo, Japan
Silica gel	E. Merck, Darmstadt, Fed. Rep. Germany
Soybean meal	Ogawa Sangyo, Co., Ltd., Tokyo, Japan
Thymidine	Kyowa Hakko Kogyo, Co., Ltd., Tokyo, Japan
[6-^{3}H]Thymidine (21.5 Ci/mmole)	The Radiochemical Centre, Amersham, U. K.

Continuation of Table 1

Product	Source
Tris(hydroxymethyl)aminomethane (Trizma Base, Tris)	E. Merck, Darmstadt, Fed. Rep. Germany
Triton X-100	Wako Pure Chem. Ind., Ltd., Osaka, Japan
Uracil	Kyowa Hakko Kogyo, Co., Ltd., Tokyo, Japan
[^{3}H]Uridine (21.5 Ci/mmole)	The Radiochemical Centre, Amersham, U. K.
Uridine 5'-diphosphate-N-acetylglucosamine (UDP-GlcNAc)	Sigma Chem. Co., St. Louis, U. S. A.
Uridine 5'-diphosphate-N-acetyl-D-glucosamine, [D-glucosamine-^{14}C(U)] (UDP-[^{14}C]GlcNAc) (235 mCi/mmole)	New England Nuclear, Boston, Mass., U. S. A.
Uridine 5'-diphosphate-N-acetyl-D-glucosamine, [glucosamine-6-^{3}H(N)]- (UDP-[^{3}H]GlcNAc) (6.6 Ci/mmole)	New England Nuclear, Boston, Mass., U. S. A.
Uridine 5'-monophosphate, sodium salt (UMP·Na)	Kyowa Hakko Kogyo, Co., Ltd., Tokyo, Japan
Vancomycin hydrochloride	Eli Lilly Co., Indianapolis, U. S. A.
Yeast extract	Oriental Kobo Kogyo, Co., Ltd., Tokyo, Japan

3. RESULTS

3.1. Morphological characterization of the strain producing azureomycins A and B, production, isolation and characterization of the antibiotics

3.1.1. Morphological characterization of the producing strain

The antibiotic producing actinomycete, strain AM-3696, was isolated from a soil sample collected in Kahoku-Cho, Nishimurayama-Gun, Yamagata Prefecture, Japan.

The microscopical morphology of the strain is described in this thesis, whereas cultural and physiological characteristics as well as chemical analysis of cell constituents of the strain are reported by ŌMURA et al.[19].

The substrate mycelium of the strain is zig-zag in shape (Plate 1). The strain forms a white abundant mass of aerial mycelium with long spore chains (Plate 2). Matured spore chains are straight (Plate 2, indicated with arrows) and consist of more than twenty spores with smooth surface. The spores are cylindrical. The spore is 1.1 - 3.7 x 0.4 µm in size (Plate 3). Acropetal budding is observed in the course of blastospore formation (Plate 4, indicated with arrow).

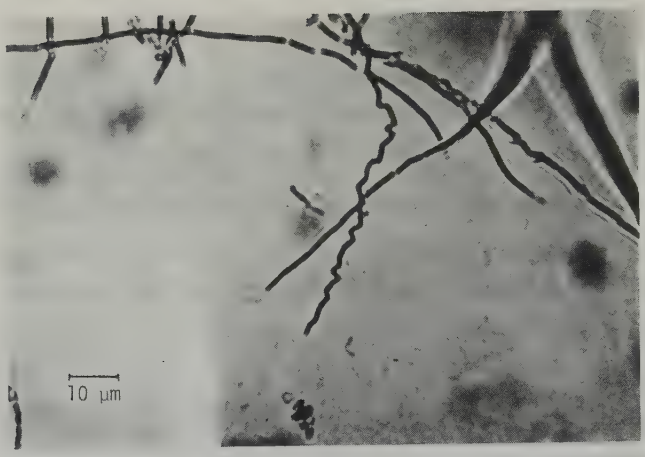


Plate 1 Substrate mycelium of strain AM-3696.
Yeast extract-malt extract agar,
4 days at 27° C.

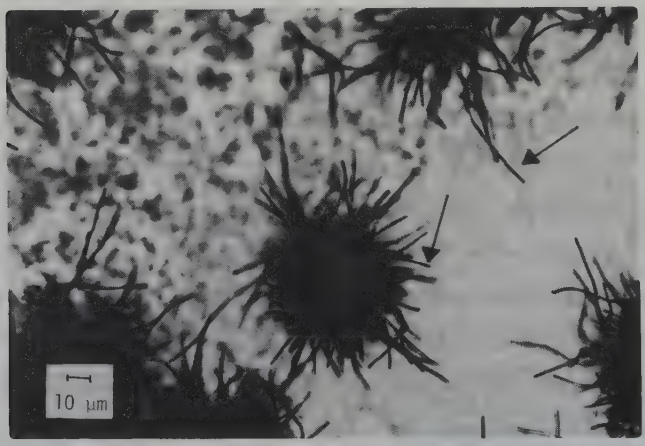


Plate 2 Aerial mycelium of strain AM-3696.
Oatmeal agar, 9 days at 27° C.
The arrows indicate straight spore chains.

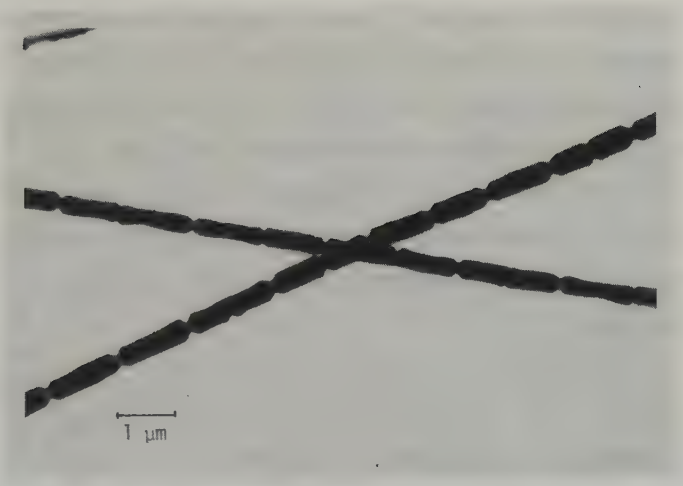


Plate 3 Spores of strain AM-3696. Yeast extract-malt extract agar, 14 days at 27° C.

Plate 4 Blastospore of strain AM-3696. Inorganic salts-starch agar, 4 days at 27° C. Acropetal budding is indicated with arrow.

According to ŌMURA et al.[19] strain AM-3696 is considered to represent a new species of the genus Pseudonocardia HENSSEN emend. HENSSEN et SCHÄFER[37], and the name Pseudonocardia azurea TAKAHASHI et ŌMURA is proposed. The epitheton "azurea" was chosen because of a characteristic soluble blue pigment produced on glucose-nitrate agar[19], and the antibiotics produced by this strain were named azureomycins.

3.1.2. Production of azureomycins A and B

The production of azureomycins A and B was carried out as described unter 2.1.2. in "Materials and Methods".

A typical time course of the production of azureomycins is given in Fig. 2. The antibiotic production started one day after the inoculation and reached a maximum level (ca. 10 µg/ml) at 4 - 5 days incubation. The antibiotic activity did not decrease until the sixth day.

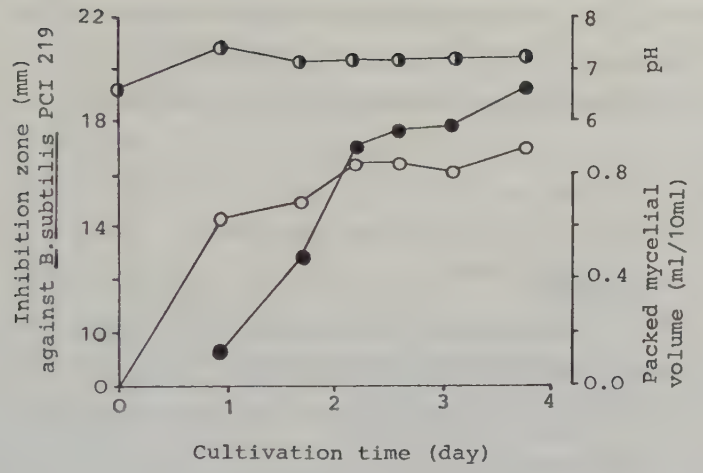


Fig. 2 Typical time-course of azureomycin production

- ● inhibition zone
- o packed mycelial volume
- ◐ pH

3.1.3. Isolation of azureomycins A and B

The isolation procedure of azureomycins A and B is shown in Fig. 3. Components A and B (yield: 100 mg and 200 mg, respectively) were separated by carboxymethyl-cellulose column chromatography. The relative amount of azureomycins A and B produced was 20 % in the case of component A and 50 % for azureomycin B of the total antibiotic activity. The remaining 30 % consist of at least 3 minor

components, the purification of which is in progress. The recovery from the culture filtrate of components A and B was 8 %. The low recovery is due to the many desalting steps necessary between each column chromatography and about 40 % loss during the isolation-steps with Avicel.

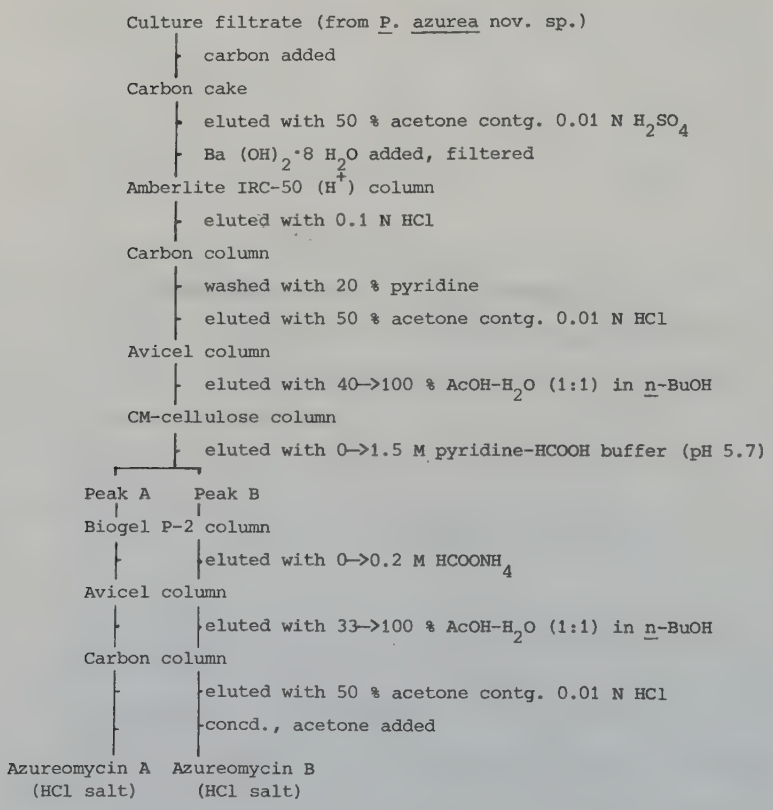

Culture filtrate (from P. azurea nov. sp.)
carbon added
Carbon cake
eluted with 50 % acetone contg. 0.01 N H_2SO_4
Ba $(OH)_2 \cdot 8 H_2O$ added, filtered
Amberlite IRC-50 (H^+) column
eluted with 0.1 N HCl
Carbon column
washed with 20 % pyridine
eluted with 50 % acetone contg. 0.01 N HCl
Avicel column
eluted with 40→100 % AcOH-H_2O (1:1) in n-BuOH
CM-cellulose column
eluted with 0→1.5 M pyridine-HCOOH buffer (pH 5.7)
Peak A
Peak B
Biogel P-2 column
eluted with 0→0.2 M $HCOONH_4$
Avicel column
eluted with 33→100 % AcOH-H_2O (1:1) in n-BuOH
Carbon column
eluted with 50 % acetone contg. 0.01 N HCl
concd., acetone added
Azureomycin A (HCl salt)
Azureomycin B (HCl salt)

Fig. 3 Isolation procedure of azureomycins A and B (HCl salts)

3.1.4. Characterization of azureomycins A and B

3.1.4.1. Physico-chemical characterization

Fig. 4 shows the ultraviolet-absorption spectra of azureomycins A and B. Absorption maxima in acidic solution were observed for azureomycin A at 282 nm and for azureomycin B at 279 nm. In an alkaline solution, shifts of absorption maxima of azureomycin A to 301 nm and of azureomycin B to 286 nm were observed. This absorption and the shift in alkaline conditions is characteristic for phenolic chromophores, which are most probably contained in both compounds.

The infrared absorption spectra in KBr (Fig. 5) show characteristic bands at 3400 cm^{-1} (amine or hydroxyl group), 1740 and 1650 cm^{-1} (carbonyl group of a peptide bond) and 1060 cm^{-1} (C-O-C stretching).

Attempts were made to determine the molecular weights by various methods including mass spectrometry, but none gave a satisfactory result. However, on the basis of the elementary analysis and the fact that 20 - 30 % of the antibiotic passed through a membrane filter DIAFLO UM-2 (Amicon, molecular weight allowance < 1000) and less than 1 % of the activity could pass through a DIAFLO UM-05 membrane (molecular weight allowance < 500), it was estimated to be around 850 $\pm$ 40 for $C_{30-34}H_{36-44}N_3O_{21-24}\cdot HCl$ for azureomycin A and 870 $\pm$ 20 for $C_{35-36}H_{46-48}N_3O_{19-21}\cdot HCl$ for azureomycin B.

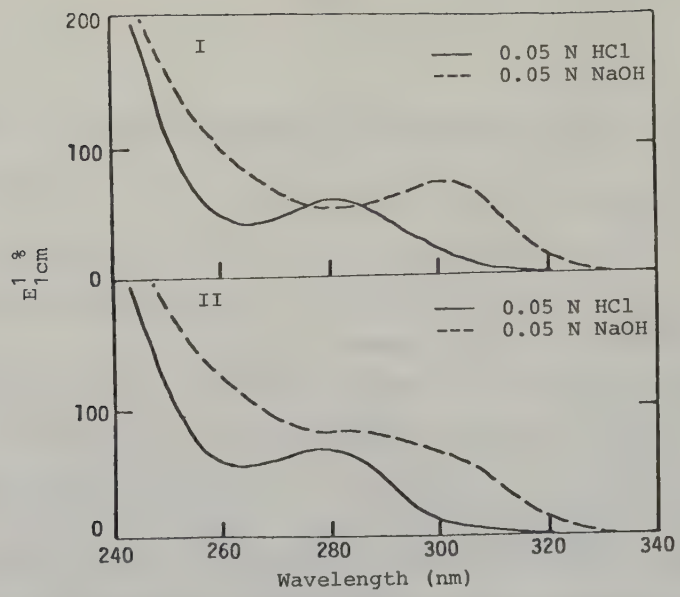


Fig. 4 Ultraviolet-absorption spectra of azureomycins A (I) and B (II) (HCl-salts).

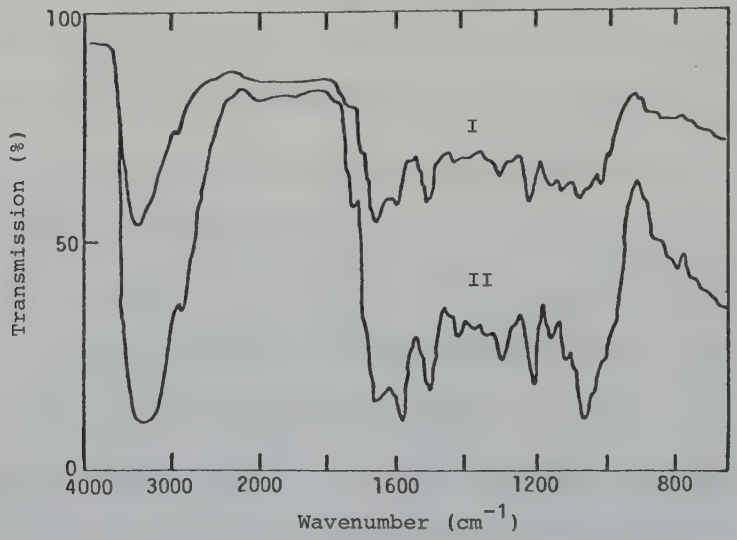


Fig. 5 Infrared-absorption spectra of azureomycins A (I) and B (II) (HCl-salts) (KBr method).

Table 2 summarizes physico-chemical properties of azureomycins A and B. Unidentified amino acids and sugars were detected by thin-layer chromatography and amino acid analysis of the acid-hydrolyzates of azureomycins A and B. The R_f values of azureomycin A and B on Avicel and silica gel thin-layer chromatography are shown in Table 3.

3.1.4.2. Biological characterization

Table 4 shows the antimicrobial spectra of azureomycins A and B. The antibiotics inhibit the growth of Gram-positive bacteria including penicillin G-resistant Staphylococcus aureus, Mycobacterium smegmatis and strains of the anaerobic genus Clostridium. But they are ineffective against Gram-negative bacteria and fungi.

No signs of toxicity were seen in mice after intraperitonial injection of 500 mg/kg of azureomycins A and B.

Table 2 Physico-chemical properties of azureomycins A and B (HCl salts)

	Azureomycin A	Azureomycin B
Anal. Found	C : 43.5 % H : 4.46 N : 4.97 Cl: 4.33	C : 47.6 % H : 5.27 N : 4.74 Cl: 3.70
MP	231° C (decomp.)	220° C (decomp.)
$[\alpha]_D^{22}$	-124° (C 0.5, water)	-116° (C 2, water)
Mol. wt.	850 ± 40	870 ± 20
Mol. Formula	$C_{30-34}H_{36-44}N_3O_{21-24}\cdot HCl$	$C_{35-36}H_{46-48}N_3O_{19-21}\cdot HCl$
UV λ_{max} $(E^{1\%}_{1cm})$		
in 0.05 N HCl	282 (54)	279 (50)
in 0.05 N NaOH	301 (81)	286 (77 sh.)
Nature	basic, colorless, water-soluble	
Color Reaction		
positive to	Rydon-Smith, Anisaldehyde-H_2SO_4, $KMnO_4$	
negative to	Elson-Morgan, Ehrlich, TTC, Sakaguchi	

Table 3 R_f values of azureomycins A and B on thin-layer chromatography

Solvent system	R_f value A	R_f value B
(Avicel)		
n-Butanol-benzene-pyridine-water (5:1:5:3, pH 5.6*)	0.34	0.15
Ethyl acetate-pyridine-water (4:3:2, pH 5.6*)	0.38	0.25
n-Butanol-acetic acid-water (1:1:1)	0.60	0.55
(Silica gel)		
Acetone-methanol-formic acid (3:1:1:1)	0.90	0.85
Ethyl acetate-pyridine-water (4:3:2)	0.45	0.48

*pH was adjusted with formic acid.

Table 4 Antimicrobial spectra of azureomycins A and B

Microorganism	MIC(μg/ml) A	MIC(μg/ml) B
Staphylococcus aureus FDA 209P	6.25	12.5
S. aureus FS 1277 (penicillin-resistant)	6.25	12.5
Bacillus subtilis PCI 219	1.56	1.56
B. cereus T	6.25	6.25
B. megaterium KM	0.39	0.10
Sarcina lutea PCI 1001	0.78	0.78
Mycobacterium smegmatis ATCC 607	12.5	12.5
Nocardia asteroides KB 54	3.13	3.13
Streptococcus pneumoniae III	3.13	1.56
Streptoc. pyogenes C203	3.13	1.56
Clostridium perfringens PB6KN5	3.13	6.25
Cl. perfringens ME 2237	1.56	6.25
Cl. botulinum IFO 3733	1.56	0.39
Cl. kainantoi IFO 3353	-	0.39
Cl. sporogenes IFO 3987	-	0.39
Escherichia coli NIHJ	> 100	> 100
Salmonella typhimurium KB 20	> 100	> 100
Pseudomonas aeruginosa P3	> 100	> 100

3.2. Mode of action of azureomycin B *in vivo*

For the investigation of the mode of action (3.2. and 3.3.) only the major component, azureomycin B, was used. As components A and B are chemically closely related substances, their mode of action will most probably not differ.

A second reason why azureomycin B was used is that it showed stronger antimicrobial activity than component A against *B. megaterium* KM, the strain which was used to determine the site of action *in vitro*.

3.2.1. Effect of azureomycin B on the growth of *B. cereus* T

When azureomycin B was added to the culture medium before inoculation, it inhibited the growth of *B. cereus* T at a concentration of 10 µg/ml (Fig. 6 A).

Upon its addition (10 µg/ml) during the log phase of the culture, growth observed as a function of decreasing light transmission seemed to continue for about 15 minutes, and then lysis occurred (Fig. 6 B).

When the viable cells in the same culture were counted, a considerable decrease was already observed after 10 minutes of incubation with azureomycin B (10 µg/ml) (Fig. 7). Upon addition of azureomycin B at the beginning of the stationary growth phase, no (Fig. 6 B) or only a weak effect on growth (Fig. 7) was seen. This observation

is common to cell wall synthesis inhibitors which act only on growing but not on resting cells.

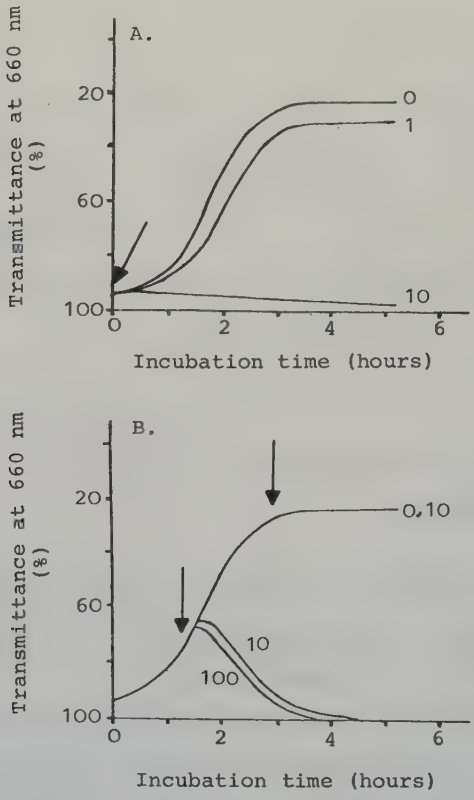


Fig. 6 Effect of azureomycin B on a growing culture of B. cereus T in nutrient broth at 37° C. The antibiotic was added at the beginning (A), at the log phase (B) and at the stationary phase (B) of incubation. The arrows indicate the addition periods of azureomycin B. The numbers in the figure show the concentration (µg/ml) of azureomycin B added to the culture.

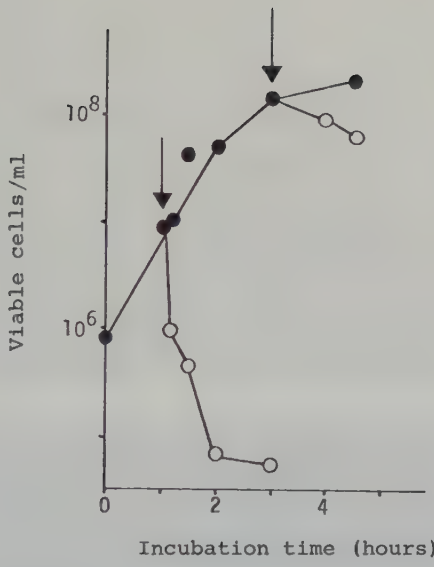


Fig. 7 Effect of azureomycin B on the viability of cells of B. cereus T.
Viable cells were counted after dilution of an aliquot with water, distribution on a nutrient agar plate and overnight incubation at 37° C. The arrows indicate the addition periods of azureomycin B.

- ● control
- o azureomycin B (10 µg/ml)

3.2.2. Morphological change of cells treated with azureomycin B

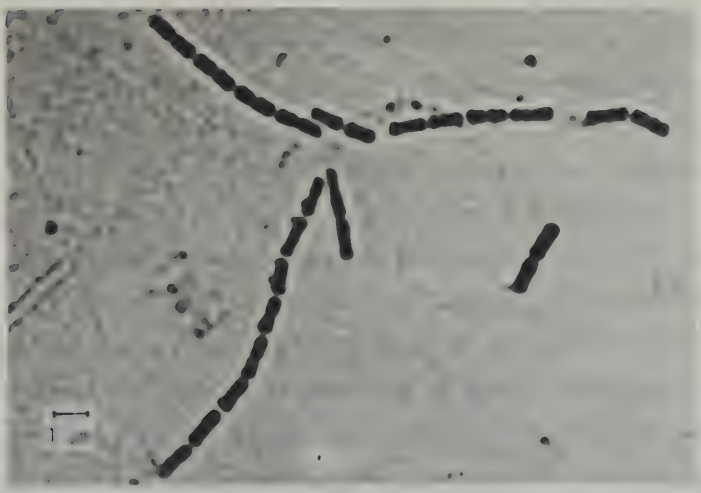
Plate 5 shows normal cells of middle log phase of B. cereus T as revealed by photomicrography.

Plate 6 shows the cells under the influence of treatment with azureomycin B (20 µg/ml) for 30 minutes. Compared to the control, the azureomycin B-treated cells appear swollen. The swollen cells then lysed (Plate 6, indicated with arrows).

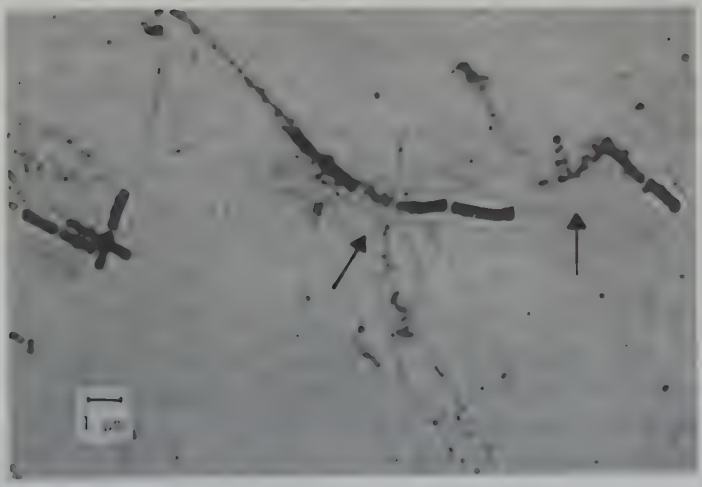
3.2.3. Effect of azureomycin B on the biosynthesis of protein, nucleic acids and cell wall peptidoglycan in B. cereus T

The incorporation of labeled precursors of protein, DNA, RNA and peptidoglycan (L-[^{14}C]leucine, [^{3}H]thymidine, [^{3}H]uridine or [^{3}H]Dpm and [^{14}C]glucosamine, respectively) into acid-insoluble macromolecular materials was studied in the presence of azureomycin B. Figs. 8, 9 and 10 show that the incorporations of [^{14}C]leucine, [^{3}H]thymidine and [^{3}H]uridine were not inhibited in the presence of 10 µg/ml of azureomycin B for at least 5 minutes, whereas typical inhibitors of protein, DNA and RNA synthesis like chloramphenicol, actinomycin D and rifampicin, respectively, inhibited the incorporation of precursors from the beginning

A.



B.



Plates 5 and 6 Middle log phase cells of B. cereus T. A, control; B, treated with azureomycin B (20 µg/ml) for 30 minutes (arrows indicate lysed cells).

of the incubation. The inhibitions observed after 10 to 20 minutes incubation are to be regarded as secondary effects derived from cease of growth and lysis of the cells which were caused by the interference of azureomycin B with peptidoglycan synthesis as shown in Figs. 11 and 12.

These figures show that the incorporations of [^{3}H]Dpm and [^{14}C]glucosamine into acid-insoluble macromolecular fraction of B. cereus T were inhibited from the beginning in the presence of 10 µg/ml of azureomycin B, the concentration which inhibited the growing culture of the organism (see Fig. 6). For 10 µg/ml azureomycin B, the extent of inhibition of [^{3}H]Dpm incorporation was 63 %, and 35 % in the case of [^{14}C]glucosamine incorporation. The weaker inhibition of [^{14}C]glucosamine incorporation can be interpreted in the way that azureomycin B affects only peptidoglycan synthesis because Dpm is a more specific precursor for it, whereas glucosamine can be easily metabolized and incorporated into other macromolecules.

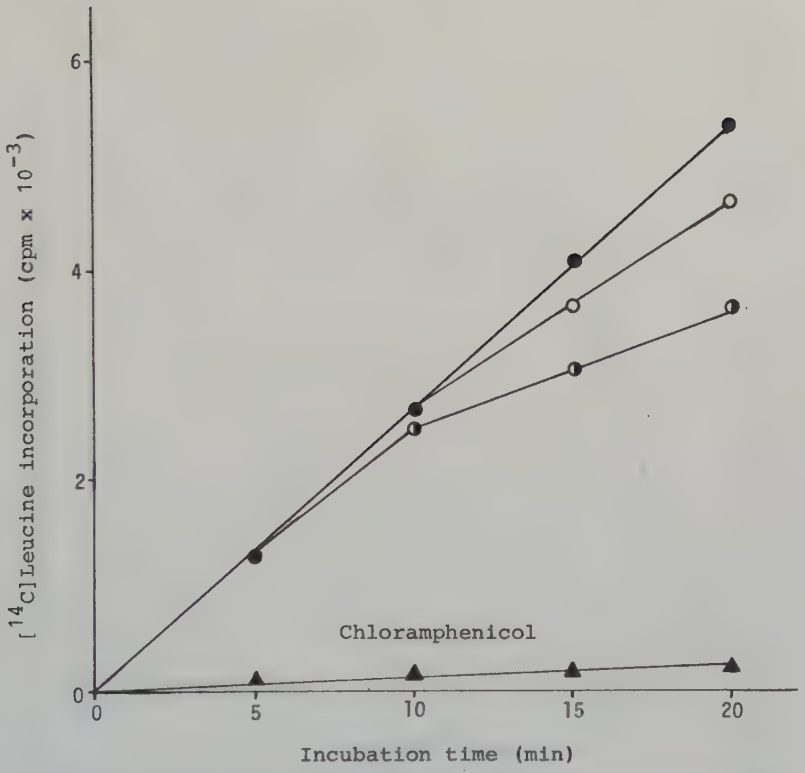


Fig. 8 Effect of azureomycin B on the incorporation of [^{14}C]leucine into acid-insoluble macromolecular fraction in B. cereus T in comparison with chloramphenicol (100 µg/ml).

- ● control
- o 10 µg/ml azureomycin B
- ◑ 50 µg/ml azureomycin B

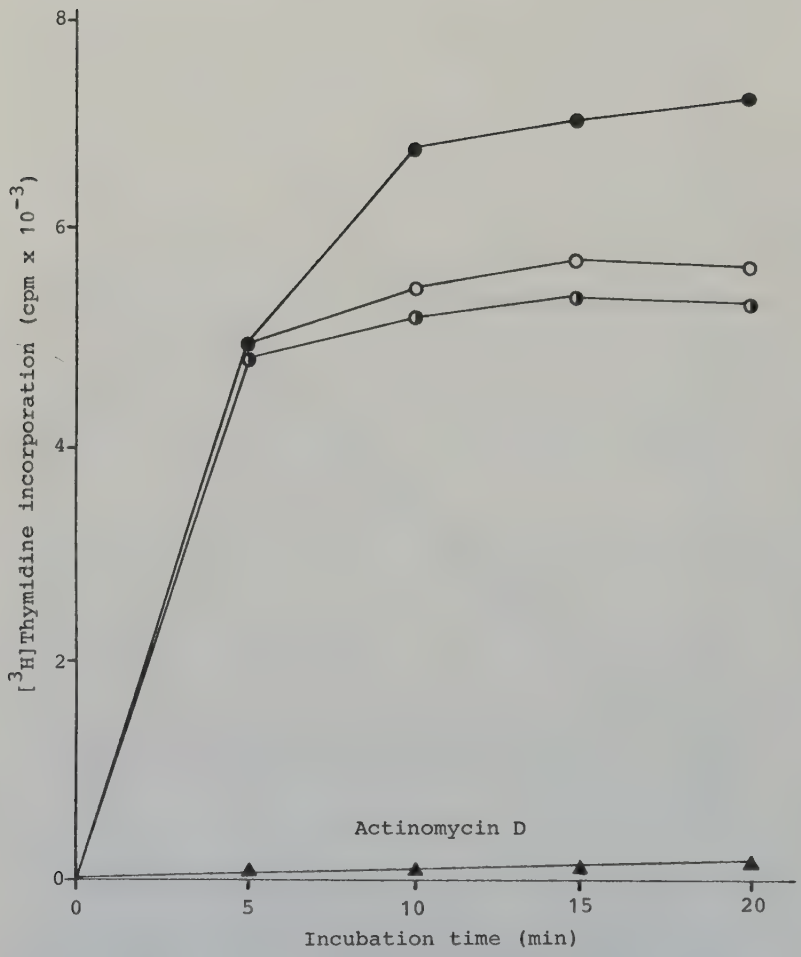


Fig. 9 Effect of azureomycin B on the incorporation of [^{3}H]thymidine into acid-insoluble macromolecular fraction in B. cereus T in comparison with actinomycin D (10 µg/ml).

- ● control
- o 10 µg/ml azureomycin B
- ◑ 50 µg/ml azureomycin B

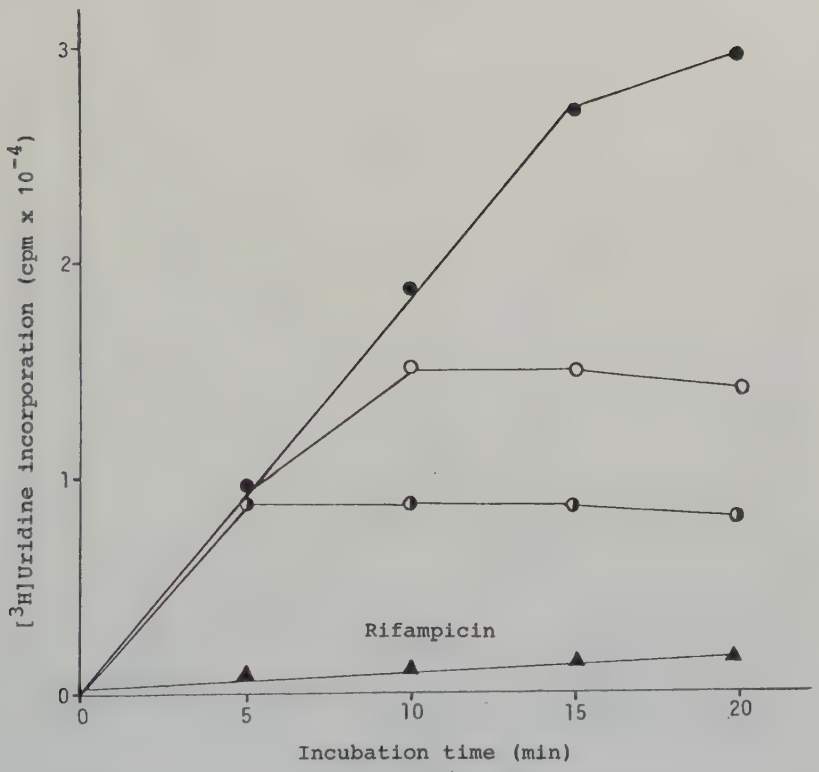


Fig. 10 Effect of azureomycin B on the incorporation of [^{3}H]uridine into acid-insoluble macromolecular fraction in B. cereus T in comparison with rifampicin (10 µg/ml).

- ● control
- o 10 µg/ml azureomycin B
- ◑ 50 µg/ml azureomycin B

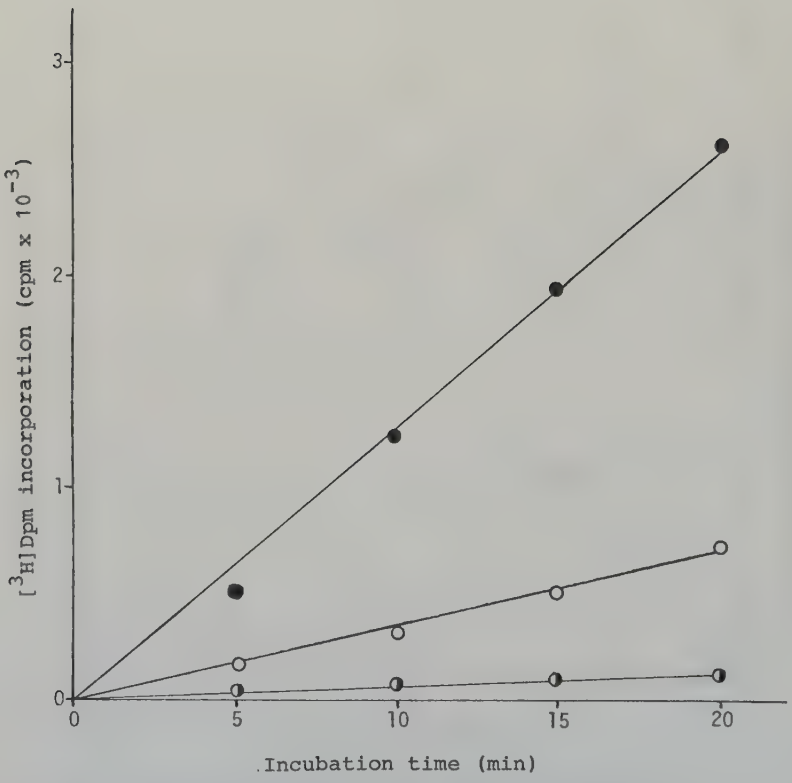


Fig. 11 Effect of azureomycin B on the incorporation of [^{3}H]Dpm into acid-insoluble macromolecular fraction in B. cereus T.

- ● control
- o 10 µg/ml azureomycin B
- ◑ 50 µg/ml azureomycin B

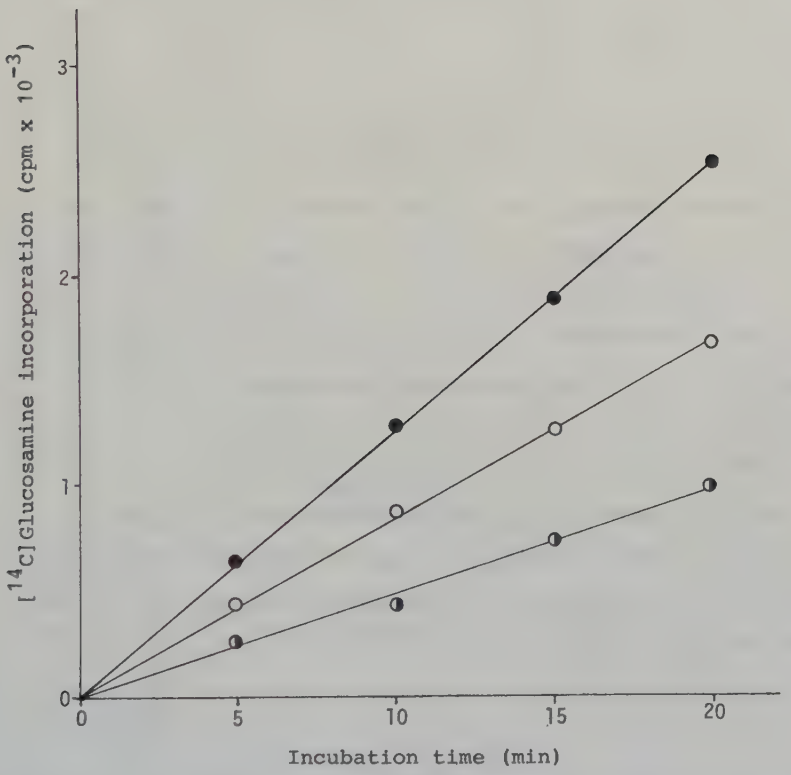


Fig. 12 Effect of azureomycin B on the incorporation of [^{14}C]glucosamine into acid-insoluble macromolecular fraction in B. cereus T.

- ● control
- o 10 µg/ml azureomycin B
- ◑ 50 µg/ml azureomycin B

3.2.4. Time course of UDP-MurNAc-peptide(s) accumulation and peptidoglycan synthesis in the absence and presence of azureomycin B in a growing culture of B. cereus T

Fig. 13 A shows peptidoglycan synthesis in the absence and presence of azureomycin B (50 µg/ml) determimed as the incorporation of [^{3}H]Dpm into acid-insoluble fraction of B. cereus T collected on a Millipore filter. In Fig. 13 B UDP-MurNAc-peptide(s) accumulation both with and without azureomycin B (50 µg/ml) is seen which was determined after paper chromatographical separation of the cell extract from an aliquot of the same culture as that used in Fig. 13 A. When the organism was incubated in the presence of azureomycin B, peptidoglycan synthesis from [^{3}H]-Dpm was strongly inhibited (Fig. 13 A) and UDP-MurNAc-peptide(s), precursor(s) of peptidoglycan, accumulated in the cells (Fig. 13 B).

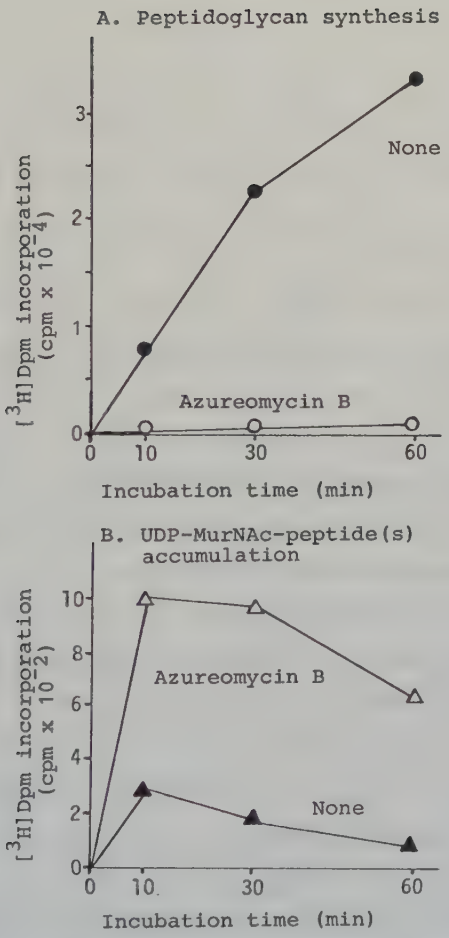


Fig. 13 Time course of peptidoglycan synthesis (A) and UDP-MurNAc-peptide(s) accumulation (B) by B. cereus T in the absence (●,▲) and presence (○,△) of azureomycin B (50 µg/ml).

3.2.5. Identification of the nucleotide precursor(s) accumulated in the cells

Figs. 14 and 15 show the column chromatograms of cold trichloroacetic acid extracts from nontreated cells (Fig. 14) and from cells incubated with azureomycin B (20 µg/ml) (Fig. 15). A large peak (fraction numbers 95 and 96) eluted with 0.1 M NaCl in 0.01 N HCl was obtained only in the antibiotic-treated culture (Fig. 15). The ultraviolet-absorption spectra of fraction 95 in acidic and alkaline conditions are given in Fig. 16. The spectra are identical with those of an authentic sample of uridine[38]. The smaller peaks at fraction numbers 45, 57, 74 and 79 in Fig. 15 having adenosine type absorption spectra were not further analyzed because the control column chromatogram (Fig. 14) showed similar peaks at fractions 51, 58, 76 and 81, and adenosine containing nucleotides have no relation to the biosynthesis of peptidoglycan.

Analysis of the UV-absorbing substance in the peak at fractions 95 and 96 of Fig. 15 indicated that it contained uridine, N-acetylhexosamine, glutamic acid, diaminopimelic acid and alanine in a molar ratio of about 1:1:1:1:3 (Table 5). This suggests that the accumulated substance is UDP-MurNAc-L-Ala-D-Glu-meso-Dpm-D-Ala-D-Ala (UDP-MurNAc-pentapeptide).

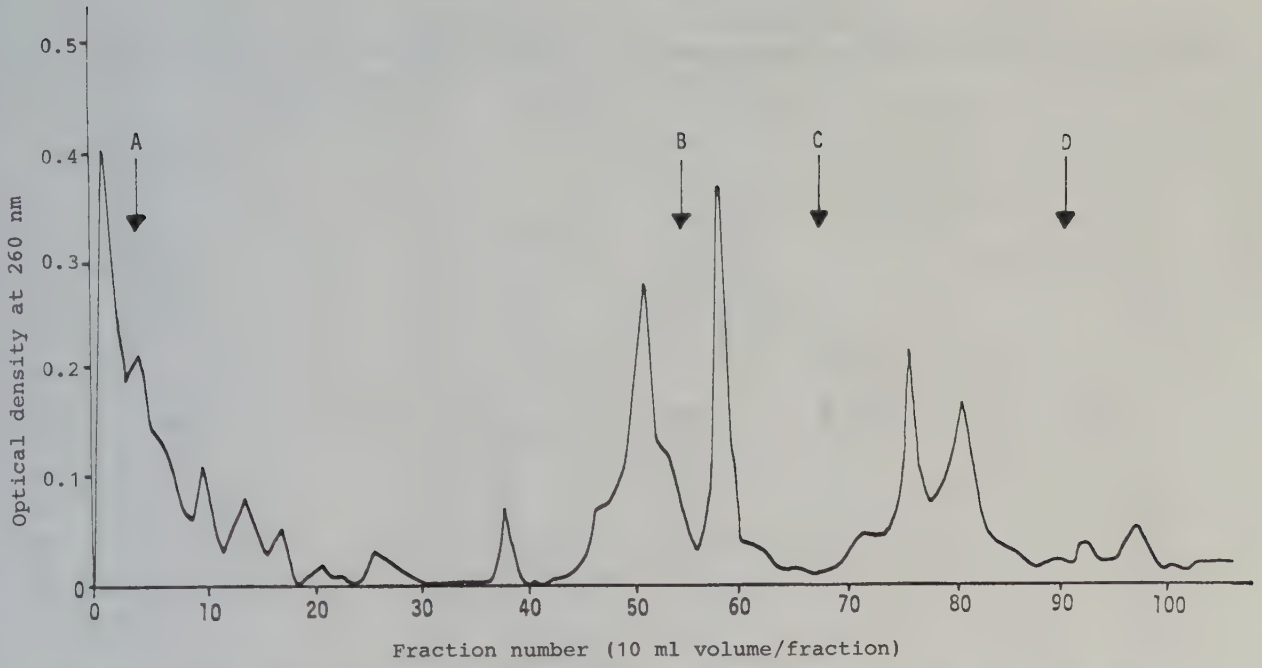
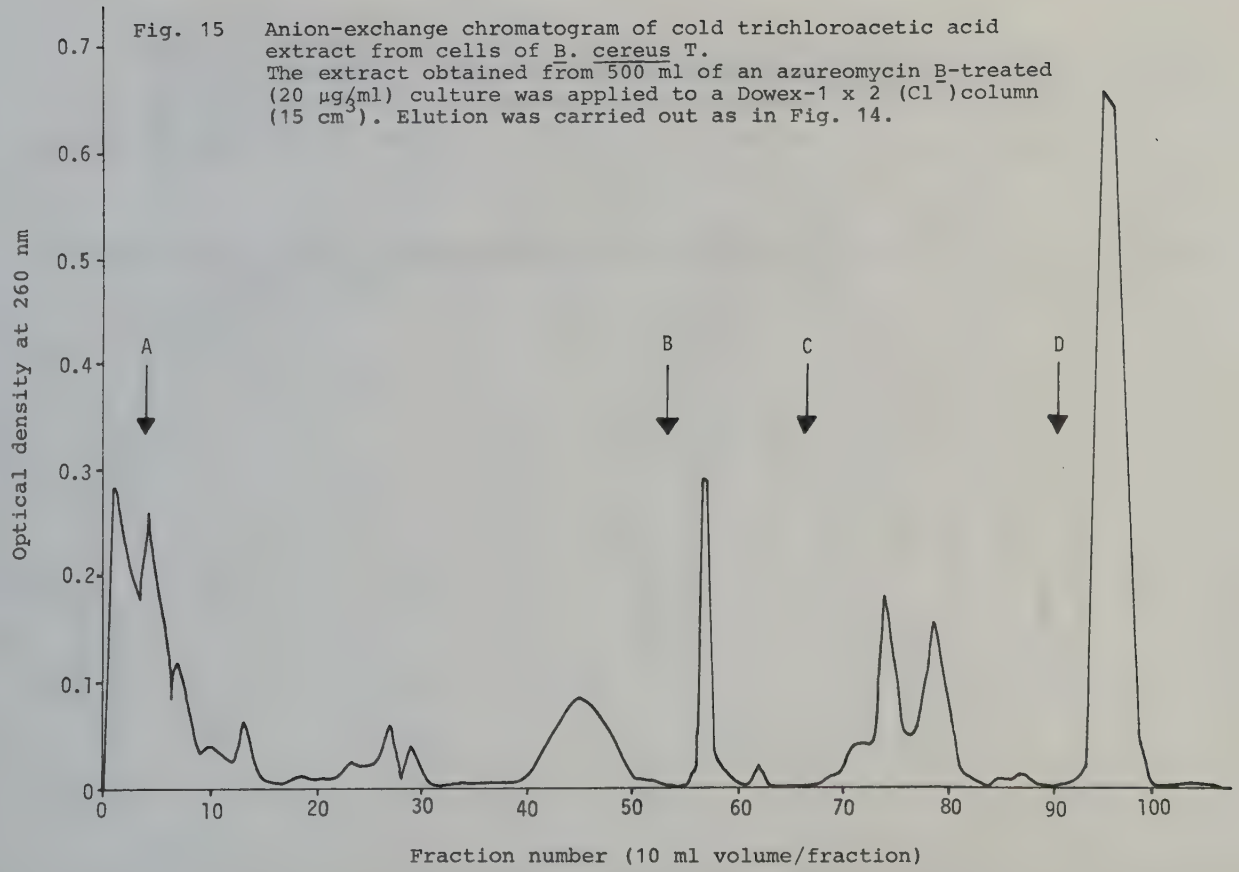


Fig. 14 Anion-exchange chromatogram of cold trichloroacetic acid extract from cells of B. cereus T.
The extract obtained from 500 ml of a control culture was applied to a Dowex-1 x 2 (Cl^-)column (15 cm^3). Elution was carried out as described by STROMINGER[25]. Eluant: A, 0.002 N HCl; B, 0.01 N HCl; C, 0.05 M NaCl in 0.01 N HCl; D, 0.1 M NaCl in 0.01 N HCl.

Fig. 15 Anion-exchange chromatogram of cold trichloroacetic acid extract from cells of B. cereus T.
The extract obtained from 500 ml of an azureomycin B-treated (20 µg/ml) culture was applied to a Dowex-1 x 2 (Cl^-) column (15 cm^3). Elution was carried out as in Fig. 14.



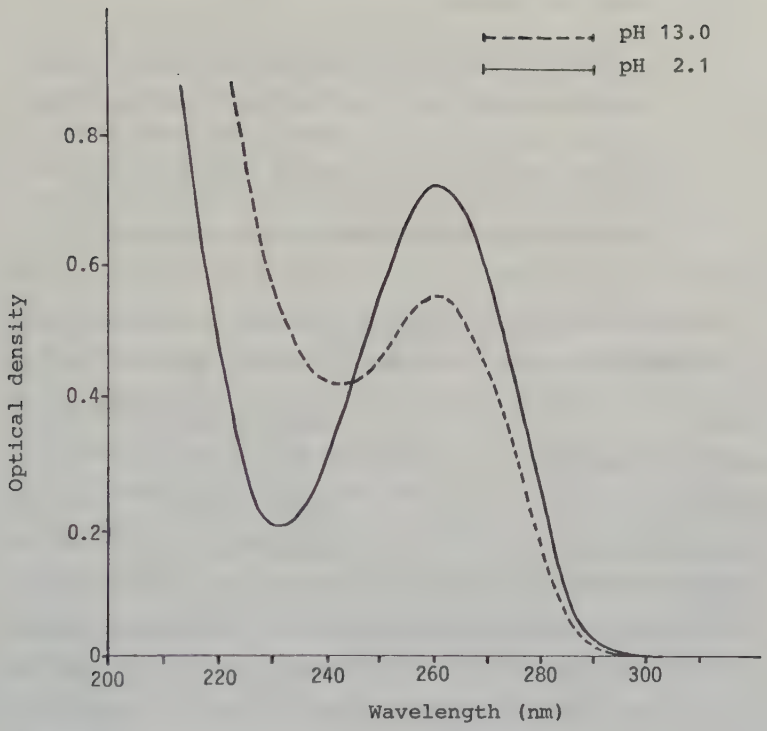


Fig. 16 Ultraviolet-absorption spectra of fraction 95 (Fig. 15) in acidic and alkaline conditions.

Table 5 Analysis of the accumulated nucleotide (fractions 95 and 96).
Both fractions were combined and further purified by charcoal adsorption and elution and by paper chromatography, and then analyzed. The analytical methods were: uridine, spectrophotometrically; GlcNAc, as N-acetylhexosamine described by REISSIG et al.[26] and STROMINGER[25]; amino acids, by amino acid analyzer after acid hydrolysis*.

Component	µmoles/ml (molar ratio)
Uridine	0.042 (1.00)
N-Acetylhexosamine	0.047 (1.12)
Glutamic acid	0.038 (0.91)
Diaminopimelic acid	0.032 (0.76)
Alanine	0.114 (2.71)

*The author thanks Mr. H. HIGUCHI from the Tokyo Research Laboratories of Kyowa Hakko Kogyo Co., Ltd. for the amino acid analysis.

3.3. Mode of action of azureomycin B *in vitro*

3.3.1. Effect of azureomycin B on the growth of *B. megaterium* KM

To investigate the mode of action of azureomycin B *in vitro*, particulate enzyme from *B. megaterium* KM was used. The membrane-bound enzymes of this strain are known to have a high activity *in vitro*[27,31]. Therefore, the effect of azureomycin B on a growing culture of *B. megaterium* KM was studied first. Fig. 17 shows that when azureomycin B was added prior to inoculation, 0.5 µg/ml of the antibiotic inhibited the growth completely. When it was added during the early log phase, 1 µg/ml of azureomycin B inhibited growth and caused lysis. Upon its addition during the late log phase, 2 µg/ml of azureomycin B caused inhibition of growth and gradual lysis. When the antibiotic was added at the beginning of the stationary phase, the cell density decreased only slightly.

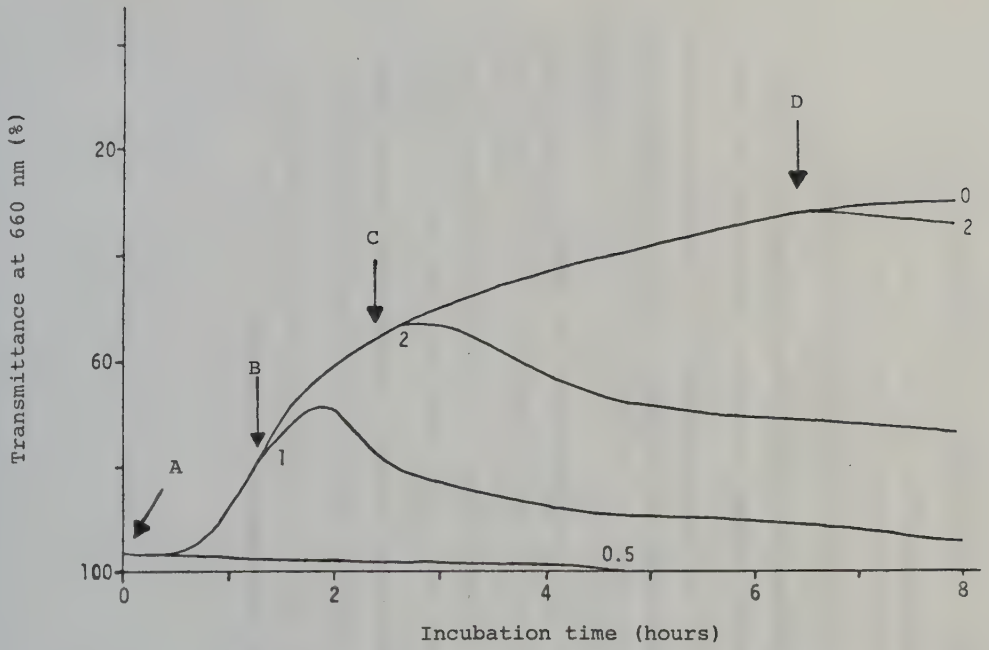


Fig. 17 Effect of azureomycin B on a growing culture of B. megaterium KM in nutrient broth at 37° C.
The antibiotic was added at the beginning (A), at the middle log phase (B), at the late log phase (C) and at the stationary phase (D) of incubation. The arrows indicate the addition period of azureomycin B. The numbers in the figure show the concentrations (µg/ml) of azureomycin B added to the culture.

3.3.2. Effect of azureomycin B on lipid intermediate accumulation and peptidoglycan synthesis with particulate enzyme from B. megaterium KM and UDP-MurNAc-[^{3}H]pentapeptide and UDP-GlcNAc as substrates

Table 6 shows the incorporation of [^{3}H]Dpm-labeled UDP-MurNAc-pentapeptide into lipid intermediate and peptidoglycan in the absence and presence of azureomycin B (20 and 100 µg/ml). Twenty µg/ml of azureomycin B showed inhibition of peptidoglycan synthesis by 69 % but no reduction in lipid intermediate synthesis in a 30-minute reaction with particulate enzyme from B. megaterium KM and UDP-MurNAc-[^{3}H]pentapeptide and UDP-GlcNAc as substrates. One hundred µg/ml of the antibiotic caused 76 % inhibition of peptidoglycan and 31 % inhibition of lipid intermediate synthesis.

From these data, it can be concluded that phospho-MurNAc-pentapeptide translocase (UMP) (see also Fig. 24 (1) in "Discussion") is not primarily affected by azureomycin B. Antibiotics known to be specific inhibitors of this reaction, tunicamycin[39] and amphomycin[32,40], simultaneously caused strong inhibition of lipid intermediate accumulation and peptidoglycan synthesis.

The primary target of the antibiotic is most probably the transfer reaction of the disaccharide-peptide unit from lipid-bound precursor to nascent peptidoglycan acceptor (see reaction (3), Fig. 24 in "Discussion"). The following experiments were done to provide additional evidence for

this presumption. Because no radioactively labeled UDP-MurNAc-pentapeptide was available, all further experiments were carried out with UDP-[^{3}H]GlcNAc and cold UDP-MurNAc-pentapeptide as the substrates.

Table 6 Effect of azureomycin B on the formation of lipid intermediate and peptidoglycan from UDP-MurNAc-L-Ala-D-Glu-meso-[^{3}H]Dpm-D-Ala-D-Ala* and UDP-GlcNAc by particulate enzyme from B. megaterium KM.
The reaction mixture A (2.3.4.) was used. The reaction time was 30 minutes.

Azureomycin B (µg/ml)	Lipid intermediate cpm (%)	Peptidoglycan cpm (%)
0	2408 (100)	1124 (100)
20	2513 (104)	343 (31)
100	1665 (69)	273 (24)

*I thank Dr. T. OKA for a sample of UDP-MurNAc-[^{3}H]pentapeptide.

3.3.3. Determination of the optimal conditions in the reaction with particulate enzyme from B. megaterium KM and UDP-[^{3}H]GlcNAc and cold UDP-MurNAc-pentapeptide as substrates

To determine the optimal conditions for the enzymatic reaction, the concentrations of particulate enzyme from B. megaterium KM (Fig. 18), UDP-MurNAc-pentapeptide (Fig. 19) and UDP-[^{3}H]GlcNAc (Fig. 20) were changed and lipid intermediate accumulation and peptidoglycan synthesis were measured.

The optimal concentration of particulate enzyme was determined as 335 µg/ml of protein (13.4 µg of protein per 40 µl-reaction mixture) (Fig. 18). The optimal UDP-MurNAc-pentapeptide concentration was 3.75×10^{-6} M (Fig. 19) and that of UDP-[^{3}H]GlcNAc was 3.75×10^{-7} M (Fig. 20).

An excess of UDP-MurNAc-pentapeptide compared to UDP-GlcNAc is probably necessary for optimal reaction, because the equilibrium of the translocase reaction (see reaction (1) Fig. 24 in "Discussion") may decline towards UDP-MurNAc-pentapeptide. The same phenomenon was also observed by STRUVE et al.[33] in case of the S. aureus Copenhagen translocase. They showed directly that UDP-MurNAc-pentapeptide has a higher affinity to the translocase than UMP.

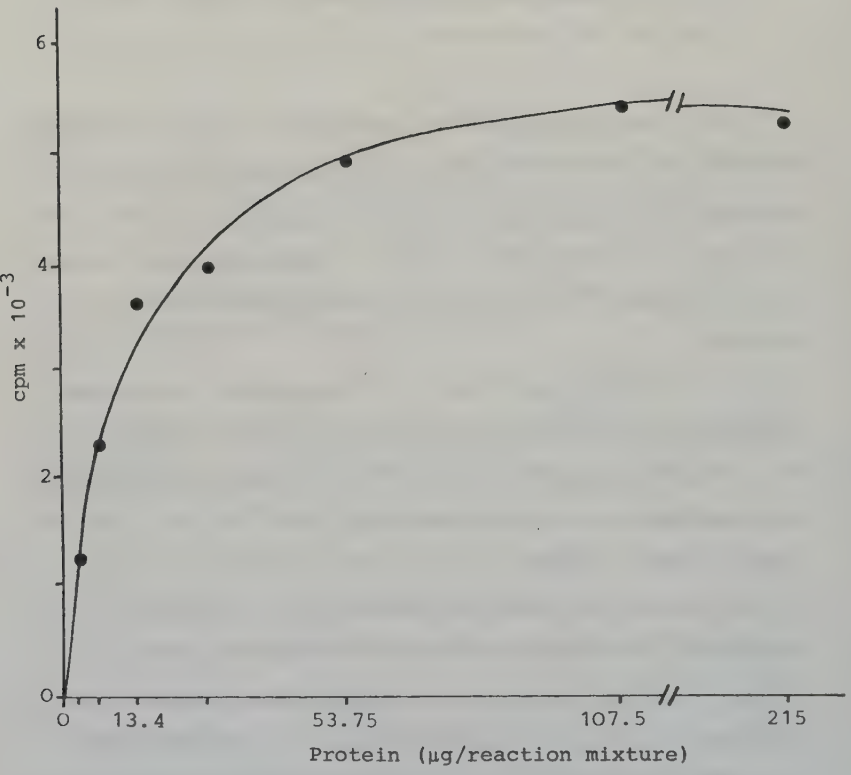


Fig. 18 Effect of the concentration of particulate enzyme from B. megaterium KM on peptidoglycan synthesis. The standard reaction mixture B (2.3.4.) was used, except that the enzyme concentration was changed. The reaction time was 2 hours. Peptidoglycan synthesis was measured as trichloroacetic acid-insoluble material after extraction of the lipid intermediate twice with 100 µl n-butanol each.

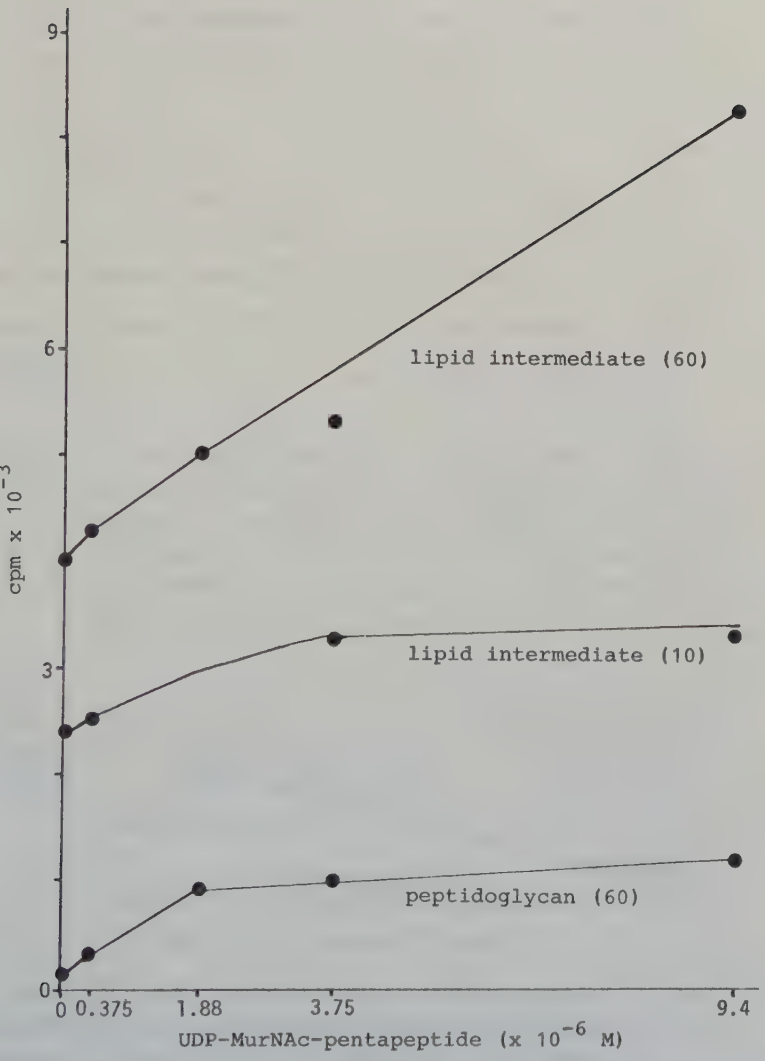


Fig. 19 Effect of cold UDP-MurNAc-pentapeptide concentration on lipid intermediate accumulation and peptidoglycan synthesis by particulate enzyme from B. megaterium KM.
The standard reaction mixture B (2.3.4.) was used, except that the concentration of UDP-MurNAc-pentapeptide was changed. The numbers in the figure indicate the time of incubation in minutes.

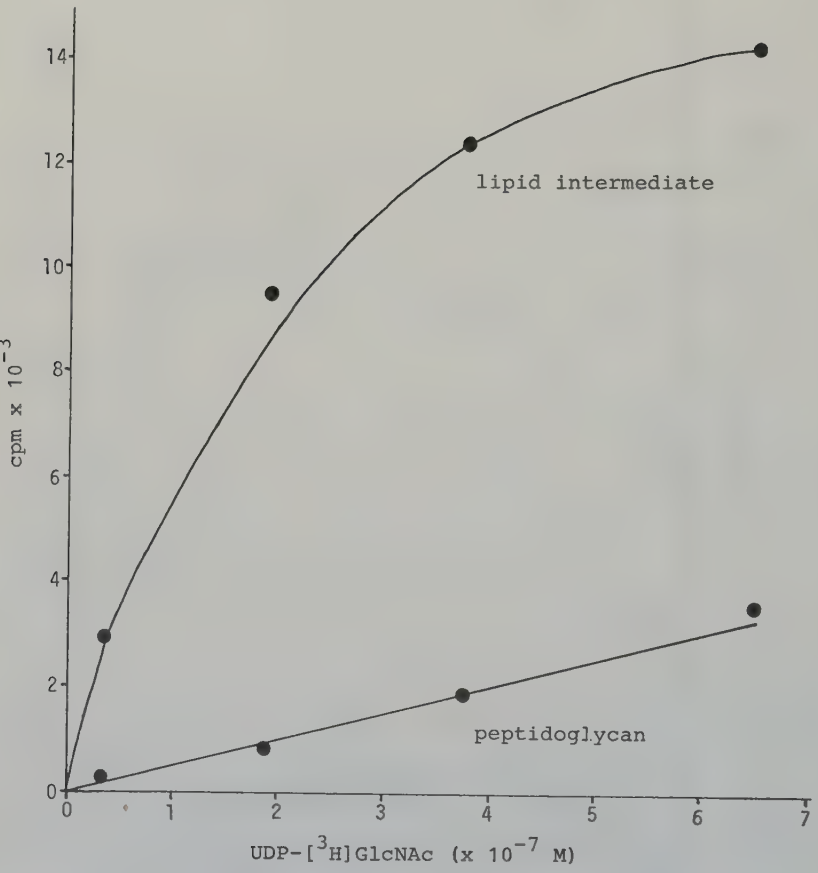


Fig. 20 Effect of the concentration of UDP-[^{3}H]GlcNAc on lipid intermediate accumulation and peptidoglycan synthesis by particulate enzyme from B. megaterium KM.

The standard reaction mixture B (2.3.4.) was used, except that the concentration of UDP-[^{3}H]GlcNAc was changed. The reaction time was 60 minutes.

3.3.4. Effect of azureomycin B on the formation of lipid intermediate from UDP-[^{3}H]GlcNAc and undecaprenol phosphate

UDP-[^{3}H]GlcNAc is used as the labeled substrate for peptidoglycan synthesis. The [^{3}H]GlcNAc or phosphate-[^{3}H]-GlcNAc moiety[41] can also be directly transferred to lipid-phosphate (Table 7) and may be utilized for teichoic acid synthesis.

Table 7 Effect of azureomycin B on the formation of lipid intermediate from UDP-[^{3}H]GlcNAc and undecaprenol phosphate from B. megaterium KM. The reaction mixture B with water instead of UDP-MurNAc-pentapeptide (2.3.4.) was used. The reaction time was two hours.

Azureomycin B (µg/ml)	n-Butanol-extractable material, cpm (%)	Trichloroacetic acid-insol. mat. cpm (%)
0	4407 (100)	249 (100)
50	4007 (91)	196 (79)

To obtain a clear result on the mode of inhibition of peptidoglycan synthesis by azureomycin B using UDP-[^{3}H]-GlcNAc as the labeled precursor, the formation of lipid intermediate from this precursor and lipid-phosphate should not be affected.

As the particulate enzyme preparation contains also the lipid-bound form of phospho-MurNAc-pentapeptide (undecaprenol-P-P-MurNAc-pentapeptide), and it was intended to examine whether azureomycin B affects the formation of lipid intermediate from UDP-[^{3}H]GlcNAc and lipid-phosphate directly as for example tunicamycin[42] does, the removement of lipid-soluble phospho-MurNAc-pentapeptide was necessary for a clear result.

HAMMES et al.[29] reported an efficient removement of the endogenous phospho-MurNAc-pentapeptide by UMP for the S. aureus Copenhagen particulate fraction (see also 2.3.2.3. in "Materials and Methods"). Here, too, this was observed by the fact that the amount of trichloroacetic acid-insoluble material decreased for 75 % after UMP-treatment of the B. megaterium KM particulate fraction, in a reaction where no cold UDP-MurNAc-pentapeptide was added.

Table 7 shows that a relative high concentration of azureomycin B (50 µg/ml) had almost no effect on the formation of lipid intermediate from UDP-[^{3}H]GlcNAc and lipid-phosphate.

This result and the fact that azureomycin B does not inhibit phospho-MurNAc-pentapeptide translocase(UMP) primarily (Table 6), allowed to use UDP-[^{3}H]GlcNAc as the labeled precursor for the examination of its mode of inhibition of peptidoglycan synthesis.

3.3.5. Time course of lipid intermediate accumulation and peptidoglycan synthesis by particulate enzyme from B. megaterium KM in the absence and presence of azureomycin B

Fig. 21 shows a time course of lipid intermediate accumulation (which also contains the lipid intermediate formed directly from UDP-[^{3}H]GlcNAc and undecaprenol phosphate) and peptidoglycan synthesis in the absence and presence of azureomycin B (10 and 50 µg/ml). Lipid intermediate accumulation was nearly not affected by 10 µg/ml of the antibiotic, whereas peptidoglycan synthesis was reduced to less than 50 % of the control value. A high concentration of azureomycin B (50 µg/ml) caused also inhibition of lipid intermediate synthesis (about 40 %) and about 80 % inhibition of peptidoglycan synthesis.

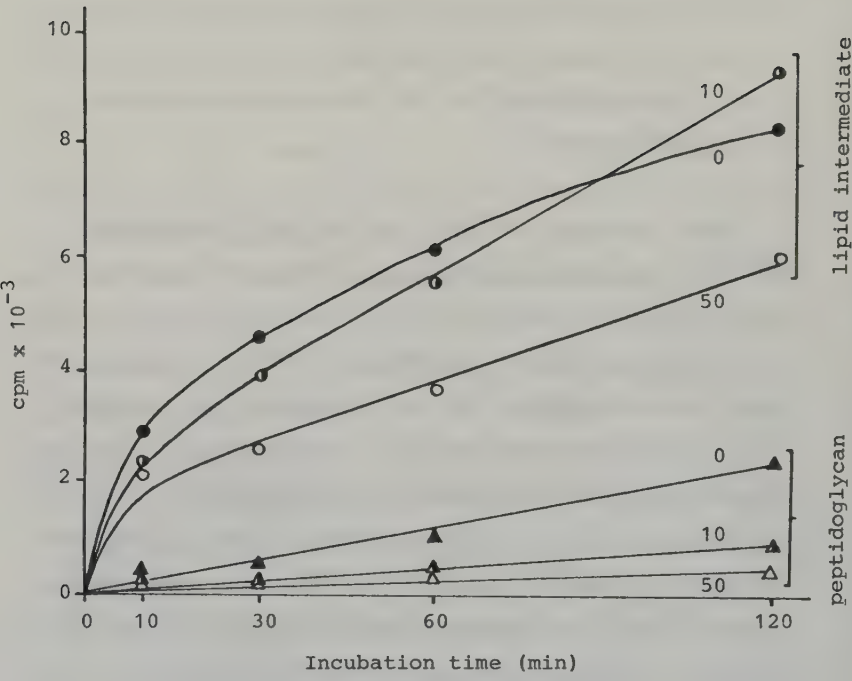


Fig. 21 Time course of lipid intermediate accumulation and peptidoglycan synthesis by particulate enzyme from B. megaterium KM in the absence and presence of azureomycin B.
The numbers in the figure indicate the concentration of azureomycin B in µg/ml. The standard reaction mixture B (2.3.4.) was used.

3.3.6. Effect of the concentration of azureomycin B on lipid intermediate accumulation and peptidoglycan synthesis by particulate enzyme from B. megaterium KM

Fig. 22 shows lipid intermediate accumulation and peptidoglycan synthesis in the dependency of increasing concentrations of azureomycin B. In a two-hour reaction, up to 10 µg/ml of the antibiotic caused accumulation of lipid intermediate and strong inhibition of peptidoglycan synthesis. This result shows clearly that the primary target of inhibition of azureomycin B is the transfer reaction of disaccharide-peptide from lipid-bound precursor to nascent peptidoglycan acceptor (Fig. 24, (3) in "Discussion") resulting in an accumulation of lipid intermediate and inhibition of peptidoglycan synthesis.

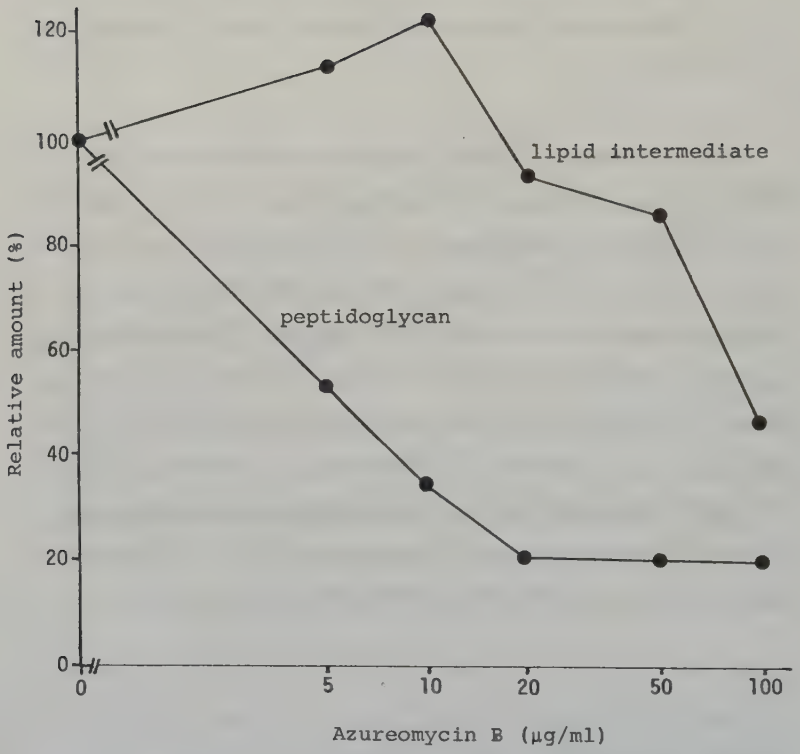


Fig. 22 Effect of the concentration of azureomycin B on lipid intermediate accumulation and peptidoglycan synthesis by particulate enzyme from B. megaterium KM.
The standard reaction mixture B (2.3.4.) was used. The reaction time was 2 hours.

3.3.7. Reversal by cell wall addition of the azureomycin B inhibition of *in vitro* peptidoglycan synthesis

It is known that the inhibition of the *in vitro* peptidoglycan synthesis by vancomycin is reversed by the addition of cell wall fraction[36]. So, the author examined whether the inhibition of *in vitro* peptidoglycan synthesis by azureomycin B could be reversed by the addition of a purified cell wall fraction from *B. megaterium* KM.

As shown in Table 8, the inhibitory effect of azureomycin B (40 μg/ml) on peptidoglycan synthesis with particulate enzyme from *B. megaterium* KM decreased to one-third in the presence of cell wall fraction (4.5 mg/ml) from the same organism. The result is in agreement with that in the case of vancomycin and it is reasonable when one thinks of the similar chemical properties of azureomycin B and vancomycin: both are sugars and amino acids containing compounds with phenolic chromophores.

Table 8 Effect of cell walls from B. megaterium KM on the inhibition of peptidoglycan synthesis by azureomycin B and vancomycin.
The reaction mixture D (2.3.4.) was used. The reaction time was two hours.

Antibiotic	Concentration (µg/ml)	Cell walls*	Peptidoglycan (cpm)	Inhibition (%)
Control		-	3142	
"		+	3035	
Azureomycin B	40	-	1158	63
"	"	+	2474	21
"	80	-	1024	67
"	"	+	2277	25
Vancomycin	16	-	723	77
"	"	+	2673	12
"	40	-	440	86
"	"	+	1902	37

* (4.5 mg/ml)

3.3.8. Binding of azureomycin B to cell walls from B. megaterium KM

An experiment based on the UV-absorbance of the antibiotic to show whether azureomycin B binds to B. megaterium KM cell walls, revealed that this binding occurred (Table 9). Eightyfour % of azureomycin B in solution were adsorbed to cell walls.

Table 9 Binding of azureomycin B (450 µg) to cell walls (600 µg) from B. megaterium KM.
The assay procedure is described in "Materials and Methods" (2.3.6.).

Cell walls	OD_{280}	Azureomycin B adsorbed (µg)
-	0.643	0
+	0.100	378

The effect of Mg^{++} on this adsorption was examined. As shown in Table 10, this cation had only a weak effect on the reduction of this binding in the case of azureomycin B and also vancomycin. It is much less than reported by BEST et al.[35] for the Mg^{++} and Mn^{++} reduction of the vancomycin binding to B. subtilis cell walls, and also less than reported by SINHA et al.[36] for the reduction of the vancomycin binding to Micrococcus lysodeicticus cell walls.

Table 10 Effect of Mg^{++} on the binding of azureomycin B (188 µg) and vancomycin (188 µg) to cell walls from B. megaterium KM.
The procedure is described in "Materials and Methods" (2.3.6.).

Antibiotic	Cell walls (µg)	OD_{280} (%)	
		$-MgCl_2$	$+MgCl_2$
Azureomycin B	-	0.668(100)	0.664(100)
"	150	0.411(62)	0.468(71)
"	300	0.123(18)	0.222(33)
Vancomycin	-	0.546(100)	0.541(100)
"	150	0.355(65)	0.456(84)
"	300	0.228(42)	0.323(60)

3.3.9. Effect of azureomycin B and vancomycin on lipid intermediate accumulation and peptidoglycan synthesis by particulate enzyme from E. coli Y-10

The optimal concentration of particulate enzyme was determined first in a similar way as in the case of the B. megaterium KM particulate (3.3.3.).

Azureomycin B did not affect the growth of Gram-negative bacteria at a concentration of 100 µg/ml (Table 4). But in an in vitro reaction using particulate enzyme from E. coli Y-10, peptidoglycan synthesis was strongly inhibited at a concentration of 10 µg/ml of azureomycin B and accumulation of lipid intermediate was caused (Table 11; Fig. 23).

Table 11 Effect of azureomycin B and vancomycin on lipid intermediate accumulation and peptidoglycan synthesis by particulate enzyme from E. coli Y-10. The reaction mixture C (2.3.4.) was used. The reaction time was two hours.

Antibiotic (µg/ml)	Peptidoglycan cpm (%)	Lipid intermediate, cpm (%)
Control (0)	1947 (100)	2350 (100)
Azureomycin B (10)	705 (36)	2822 (120)
Azureomycin B (100)	221 (11)	3118 (133)
Vancomycin (10)	1138 (59)	2082 (89)
no cold UDP-MurNAc-pentapeptide added (0)	205 (11)	1111 (47)

Vancomycin (10 µg/ml), which in vivo also inhibits Gram-positive bacteria only, reduced the in vitro peptidoglycan synthesis too, although not as much as azureomycin B did (Table 11). When cold UDP-MurNAc-pentapeptide was not added to the reaction mixture, peptidoglycan synthesis was 11 % and lipid intermediate synthesis 47 % of the control value. As the particulate enzyme preparation of E. coli Y-10 was not treated with UMP, phospho-MurNAc-pentapeptide is contained in the membrane fraction and can be utilized for

lipid intermediate and peptidoglycan syntheses. So, without cold UDP-MurNAc-pentapeptide added, a small amount of peptidoglycan and lipid intermediate can be formed.

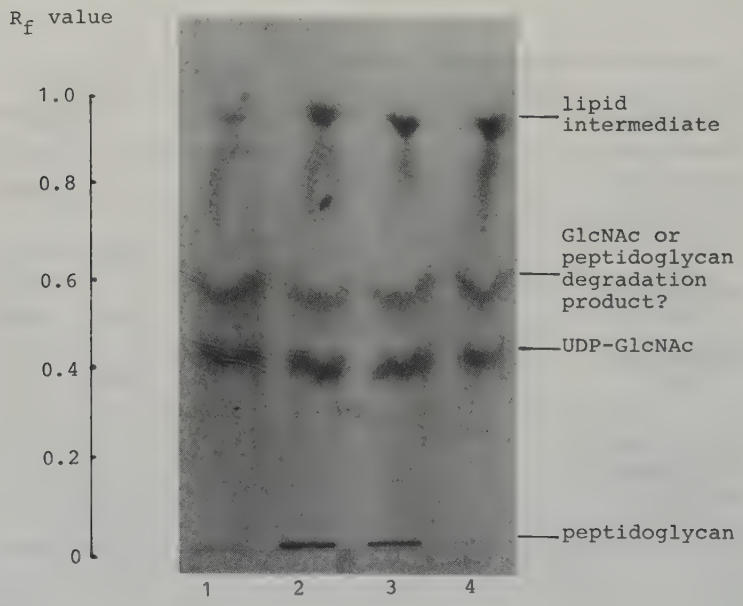


Fig. 23 Effect of azureomycin B on lipid intermediate accumulation and peptidoglycan synthesis by particulate enzyme from E. coli Y-10.

1. no cold UDP-MurNAc-pentapeptide added
2. control
3. azureomycin B (10 µg/ml)
4. azureomycin B (100 µg/ml)

The reaction mixture C (2.3.4.) was used. After 2 hours incubation at 25° C, 25 µl aliquots of each sample were spotted on paper and developed in isobutyric acid : 1 N ammonia = 5:3 for 16 hours. An autoradiogram of the chromatogram is shown.

4. DISCUSSION

4.1. Azureomycins A and B and other chemically related compounds

Azureomycins A and B were found in the culture filtrate of a new actinomycete designated as Pseudonocardia azurea TAKAHASHI et ŌMURA nov. sp.

Three species had formerly been reported to belong to the genus Pseudonocardia HENSSEN emend. HENSSEN et SCHÄFER[37]: P. thermophila[37,43,44], P. spinosa[37] and P. fastidiosa[45]. Among them, only P. fastidiosa is known to be an antibiotic producer[45].

Azureomycins A and B are basic water-soluble compounds with ultraviolet absorption maxima at around 280 nm. They are active against Gram-positive bacteria including Mycobacterium and Clostridium in a concentration range of 0.1 to 12.5 µg/ml. Gram-negative bacteria and fungi were not inhibited.

Azureomycins A and B were compared with known antibiotics from actinomycetes[46,47], other bacteria and fungi. Among them, avoparcin (LL-AV 290)[48], vancomycin[49], enduracidin[13], triculamine[50], antibiotic A-4696[51], antibiotic A-477[52], antibiotic AM-374[53] and antibiotic OA-7653[54] showed some resemblance to azureomycins A and B. But all these compounds are different from azureomycins A and B in UV spectra in acidic and alkaline solutions, elementary com-

position, molecular weights and other characteristics. Consequently, azureomycins A and B are considered to be new antibiotics.

UV and IR spectra and color reactions suggest that azureomycins A and B are sugar peptide antibiotics with phenolic chromophores.

4.2. Mode of action of azureomycin B *in vivo*

a) Effect on growth

Azureomycin B inhibited the growing cultures of *B. cereus* T and *B. megaterium* KM when added to the culture medium before inoculation and during the log phase of the culture. When added during the early stationary phase, azureomycin B caused only a very weak inhibition (3.2.1. and 3.3.1.). This observation is common to cell wall synthesis inhibitors which act only on growing but not on resting cells.

b) Morphological change of cells

When cells of middle log phase of *B. cereus* T were treated with azureomycin B, the cells showed swelling before lysis occurred (3.2.2.). Morphological change is cha-

racteristic for cell wall synthesis inhibitors and has already been demonstrated for various penicillins and cephalosporins[55], vancomycin[56] and globomycin[57]. Penicillin G, mecillinam (FL-1060) and vancomycin are well known inhibitors of peptidoglycan synthesis and, with the exception of mecillinam, are active against Gram-positive bacteria only. Globomycin, on the other hand, inhibits only Gram-negative bacteria and causes spheroplast-formation of the bacterial rods of E. coli in an isotonic medium[57]. The studies on the mode of action of this antibiotic revealed that it is a specific inhibitor of the synthesis of BRAUN's lipoprotein and gives rise to the accumulation of a precursor, prolipoprotein[58].

This lipoprotein occurs only in Gram-negative bacteria and connects covalently peptidoglycan with the outer membrane of these organisms[4,59-61].

c) Accumulation of nucleotide precursor

The selective inhibition of the incorporation of peptidoglycan precursors in the presence of the antibiotic (3.2.3.) and the accumulation of UDP-MurNAc-pentapeptide in azureomycin B-treated cells (3.2.4. and 3.2.5.), provide evidence for peptidoglycan synthesis as the target of this new antibiotic. Accumulation of nucleotide precursors in the cells is known for many other cell wall synthesis in-

hibitors like penicillin[25], vancomycin[62,63], amphomycin[40], enduracidin[64], gardimycin[65] and moenomycin-group antibiotics[66,67].

Because UDP-MurNAc-pentapeptide was accumulated in the presence of azureomycin B, a biosynthetic step after the formation of this precursor must be inhibited by the antibiotic. To clarify the site of action of azureomycin B, further *in vitro* studies (3.3.) were done to demonstrate whether the antibiotic interfers with the formation of lipid intermediate (the membrane-bound precursors of peptidoglycan), with the polymerization of these precursors to linear glycan strands, or with the cross-linking reaction of glycopeptide chains (the last step of peptidoglycan synthesis).

4.3. Mode of action of azureomycin B *in vitro*

a) Site of action of azureomycin B

In an *in vitro* system with particulate enzyme from *B. megaterium* KM and the substrates for peptidoglycan synthesis, UDP-MurNAc-pentapeptide and UDP-GlcNAc, azureomycin B caused accumulation of lipid intermediate and inhibition of peptidoglycan synthesis at low concentrations, up to 20 µg/ml (3.3.2. and 3.3.6.). This result leads to the transfer reaction of disaccharide-peptide from lipid-bound

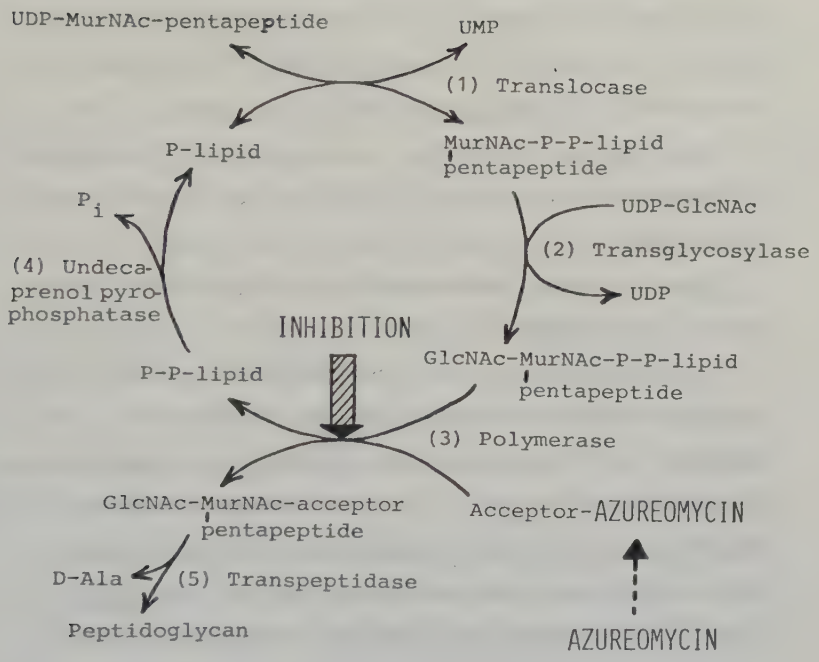


Fig. 24 Pathway of peptidoglycan synthesis from UDP-MurNAc-pentapeptide and UDP-GlcNAc in B. megaterium KM and primary site of inhibition of azureomycin B.

precursor to nascent peptidoglycan acceptor (Fig. 24 (3)) as the primary inhibitory target of azureomycin B. This acceptor is in vivo probably the growing point of a peptidoglycan chain[31,68], but in vitro it is possible that other molecules[31,69] (for example the undecaprenol phosphate carrier itself[70,71]) function as acceptor.

Antibiotics known to cause both accumulation of lipid intermediate and inhibition of peptidoglycan synthesis *in vitro* are moenomycin[72], macarbomycin[67], gardimycin[65], vancomycin[72,73] and ristocetin[73].

When particulate enzyme from *E. coli* Y-10 was used, peptidoglycan synthesis, too, was strongly inhibited with concomitant accumulation of lipid intermediate (3.3.9.).

The reason why azureomycins A and B are ineffective against Gram-negative bacteria *in vivo* (3.1.4.2.) is that these bacteria have on outer membrane which functions as permeability barrier to many substances (resistance of penetration)[74]. Although usually hydrophilic antibiotics, with a molecular weight lower than 600-900, are quite effective against Gram-negative bacteria because they can pass through water containing pores of the outer membrane[74], the molecular weights of azureomycins A and B (around 850) seem to be too high for this passage.

b) Mechanism of inhibition of polymerization by azureomycin B

The known inhibitors of the polymerization reaction mentioned above (moenomycin, macarbomycin, vancomycin etc.), however, differ in their mechanism of inhibition of the polymerase (Fig. 24, (3)). While the vancomycin and the ristocetin inhibition of peptidoglycan synthesis can be re-

versed by the addition of cell walls[36], in the case of macarbomycin this is not possible[67]. Therefore, the effect of cell walls on the inhibition of peptidoglycan synthesis was also studied in the case of azureomycin B to decide whether its mechanism of inhibition resembles the "vancomycin-type" or the "macarbomycin-type" of inhibition. The result (3.3.7.) showed that reversal of the inhibition of peptidoglycan synthesis by cell walls occurred. Regarding the chemical relationship between vancomycin and azureomycin B, this is not surprising. It is interesting, however, that azureomycin B having half the molecular weight of vancomycin (about 1600[68]) exerts a similar (if not the same) mechanism of inhibition.

The direct binding of azureomycin B to cell walls from B. megaterium KM (3.3.8.) showed even more the close relationship to vancomycin. The phenolic chromophore(s) in azureomycin B may be involved in this binding as it is true for vancomycin[68,75].

As peptidoglycan synthesis measured as trichloroacetic acid-precipitable material was markedly inhibited by azureomycin B, the inhibition of the cross-linking reaction of peptidoglycan strands (Fig. 24, (5)), as penicillins and cephalosporins do[76], can be excluded.

If the undecaprenol pyrophosphatase (Fig. 24, (4)) would be inhibited as in the case of bacitracin[77], no accumulation of lipid intermediate would be observed.

From these data and the discussion described above it is reasonable to conclude that azureomycin B inhibits the polymerization step by binding to the acceptor (Fig. 24) which *in vivo* is most probably nascent peptidoglycan. As a result, further synthesis of linear peptide strands becomes impossible.

c) Other possible sites of action of azureomycin B

At higher concentrations of azureomycin B (20 µg/ml upwards), the lipid intermediate synthesis by particulate enzyme from *B. megaterium* KM was markedly inhibited (3.3.2., 3.3.5. and 3.3.6.). If azureomycin B binds, as proposed for vancomycin[78-80], to the D-alanyl-D-alanine terminus of the peptide subunit occurring in the peptidoglycan, its lipid-bound precursors and UDP-MurNAc-pentapeptide, then this inhibition of lipid intermediate synthesis becomes reasonable. BEST et al.[81], however, showed that vancomycin and ristocetin have a higher affinity for cell walls than for UDP-MurNAc-pentapeptide. STRUVE et al.[33] and SINHA et al.[36] examined directly the effect of vancomycin on the phospho-MurNAc-pentapeptide translocase (UMP) (Fig. 24, (1)) and demonstrated that the exchange reaction of [^{3}H]UMP with the UMP-moiety of UDP-MurNAc-pentapeptide by the enzyme was inhibited.

Inhibition of the translocase at higher concentrations of azureomycin B is also possible as in the case of vancomycin and would explain in part the decrease of lipid intermediate synthesis in the presence of higher antibiotic concentrations (Table 6, Figs. 21 and 22). In fact, as also mentioned by HAMMES et al.[71] for vancomycin, *in vivo* and *in vitro* mode of action of azureomycin B may well be different. It seems more reasonable that *in vivo* a very hydrophilic compound as azureomycin B does not exert its primary inhibitory effect in the lipophilic cytoplasmic membrane, to which a major part of it probably does not penetrate, but that the hydrophilic cell wall is more easily accessible to it. The binding of the basic azureomycin B molecules to the acidic cell wall polymers of *B. megaterium* KM provides additional evidence for this hypothesis (3.3.8.).

Inhibition of the D-alanine carboxypeptidase from various bacteria is reported for vancomycin[82-84]. If azureomycin B binds to the D-alanyl-D-alanine end of uncross-linked peptidoglycan, the release of the last alanine molecule, catalyzed by the D-alanine carboxypeptidase, becomes impossible.

All experiments concerning the mode of action were done with the major component produced, azureomycin B. Because azureomycin A is chemically closely related to component B, it is reasonable to conclude that its mode of action will not differ. Both components can be regarded as new inhibitors of bacterial cell wall peptidoglycan synthesis.

5. SUMMARY

New basic, water-soluble antibiotics, azureomycins A and B, were isolated from the culture broth of an actinomycete, strain AM-3696, which was designated as Pseudonocardia azurea nov. sp.

The antibiotics contain sugar and amino acid moieties and phenolic chromophore(s). They are active against Gram-positive bacteria including a penicillin-resistant strain of Staphylococcus, and strains of Clostridium and Mycobacterium.

Azureomycin B is a specific inhibitor of peptidoglycan synthesis in bacteria. The antibiotic lysed growing cells of B. cereus T at a concentration of 10 µg/ml but did not affect resting cells. Microscopical observation revealed swelling and lysis of the bacterial rods when treated with azureomycin B. The incorporations of [^{3}H]diaminopimelic acid and [^{14}C]glucosamine into acid-insoluble fraction of growing cells of B. cereus T were strongly inhibited in the presence of azureomycin B, but those of [^{14}C]leucine, [^{3}H]thymidine and [^{3}H]uridine were not at least until 5 minutes after the beginning of the incubation. The antibiotic caused accumulation of a nucleotide precursor in the cells which was identified as UDP-MurNAc-L-Ala-D-Glu-meso-Dpm-D-Ala-D-Ala.

In an in vitro system using particulate enzyme from B. megaterium KM and UDP-MurNAc-[^{3}H]pentapeptide and UDP-

GlcNAc or cold UDP-MurNAc-pentapeptide and UDP-[^{3}H]GlcNAc as substrates, azureomycin B caused accumulation of lipid intermediate and inhibition of peptidoglycan synthesis at concentrations up to 20 µg/ml, indicating that its primary target is the transfer reaction of disaccharide-peptide from lipid-bound precursor to nascent peptidoglycan acceptor. At higher concentrations, lipid intermediate synthesis was also inhibited. Addition of purified cell walls from B. megaterium KM to the reaction mixture containing particulate enzyme from the same organism and UDP-[^{3}H]-GlcNAc and cold UDP-MurNAc-pentapeptide, caused reversal of the azureomycin B inhibition of peptidoglycan synthesis by two-thirds. A study, undertaken to determine whether azureomycin B binds to the same cell walls, revealed that this binding occurred and that Mg^{++} reduced it to a small extent.

In a reaction with particulate enzyme from E. coli Y-10 and cold UDP-MurNAc-pentapeptide and UDP-[^{14}C]GlcNAc as substrates, peptidoglycan synthesis was strongly inhibited and lipid intermediate accumulated in the presence of azureomycin B.

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